plasminogen activity was an effective test for segregating wildtype individuals and those heterozygous for the plasminogen A620T mutation. Thus, plasma PS activity is influenced by environmental factors to a greater extent than plasminogen activity.

The environmental factors such as age, sex hormone, and inflammation, are known to influence the PS activity [19]. As shown in Fig. 3, gender- and age-related differences in PS activity were observed in the general Japanese population. In addition, plasma PS activity might be influenced by other genetic factors. Genome scan for plasma free PS levels indicated a quantitative trait locus on human chromosome 1q [20]. This region contains *C4BPA* and *C4BPB* genes that are differentially regulated by acute phase cytokines [21]. PS can bind to the β-chain of C4 binding protein and not to the α-chain. The resulting alterations in the synthesis of C4 binding protein isoforms may affect the equilibrium between bound and free PS. Alternative means must be developed for the identification of PS K196E carriers to reduce the risk of DVT in affected individuals.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Dahlback B. Blood coagulation. *Lancet* 2000; **355**: 1627–32.

2 Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, Chandy M, Dahlback B, Ginter EK, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 1. *Thromb Haemost* 1996; **76**: 651–62.

3 Bucciarelli P, Rosendaal FR, Tripodi A, Mannucci PM, De Stefano V, Palareti G, Finazzi G, Baudo F, Quintavalla R. Risk of venous thromboembolism and clinical manifestations in carriers of antithrombin, protein C, protein S deficiency, or activated protein C resistance: a multicenter collaborative family study. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1026–33.

4 Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, Tsuda H, Kang D, Hamasaki N. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005; **38**: 908–15.

5 Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, Kojima T, Murata M, Nishigami K, Chiku M, Hayashi T, Kokubo Y, Okayama A, Tomoike H, Ikeda Y, Miyata T. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006; **107**: 1737–8.

6 Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. *Blood* 1994; **83**: 683–90.

7 Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S (Tokushima) (Lys155-> Glu) for the regulation of the blood coagulation system. *Biochim Biophys Acta* 1995; **1272**: 159–67.

8 Hayashi T, Nishioka J, Suzuki K. Characterization of dysfunctional protein S-Tokushima (K155-> E) in relation to the molecular interactions required for the regulation of blood coagulation. *Pol J Pharmacol* 1996; **48**: 221–3.

9 Shigekiyo T, Uno Y, Kawauchi S, Saito S, Hondo H, Nishioka J, Hayashi T, Suzuki K. Protein S Tokushima: an abnormal protein S found in a Japanese family with thrombosis. *Thromb Haemost* 1993; **70**: 244–6.

10 Yamazaki T, Sugiura I, Matsushita T, Kojima T, Kagami K, Takamatsu J, Saito H. A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. *Thromb Res* 1993; **70**: 395–403.

11 Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis in Japanese, importance of protein S K196E mutation. *Int J Hematol* 2006; **83**: 217–23.

12 Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. *J Thromb Haemost* 2004; **2**: 1012–3.

13 Okamoto A, Sakata T, Mannami T, Baba S, Katayama Y, Matsuo H, Yasaka M, Minematsu K, Tomoike H, Miyata T. Population-based distribution of plasminogen activity and estimated prevalence and relevance to thrombotic diseases of plasminogen deficiency in the Japanese: the Suita Study. *J Thromb Haemost* 2003; **1**: 2397–403.

14 Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. *Thromb Haemost* 1995; **74**: 1271–5.

15 Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004–8.

16 Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet* 1995; **346**: 1133–4.

17 Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, Brenner B, Eldor A, Seligsohn U. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood* 1997; **89**: 397–402.

18 Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost* 1995; **74**: 1381–2.

19 Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* 2004; **103**: 1192–201.

20 Almasy L, Soria JM, Souto JC, Coll I, Bacq D, Faure A, Mateo J, Borrell M, Munoz X, Sala N, Stone WH, Lathrop M, Fontcuberta J, Blangero J. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project. *Arterioscler Thromb Vasc Biol* 2003; **23**: 508–11.

21 Garcia de Frutos P, Alim RI, Hardig Y, Zoller B, Dahlback B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 1994; **84**: 815–22.

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Review Article

Genetic Risk Factors for Deep Vein Thrombosis among Japanese: Importance of Protein S K196E Mutation

Toshiyuki Miyata,[a] Rina Kimura,[a] Yoshihiro Kokubo,[b] Toshiyuki Sakata[c]

[a]*Research Institute,* [b]*Department of Preventive Cardiology, and* [c]*Laboratory of Clinical Chemistry, National Cardiovascular Center, Suita, Osaka, Japan*

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Abstract

There is mounting evidence that mutations associated with a given disease arise with different frequencies among ethnic groups, thus ethnicity-specific studies are needed to identify causative mutations and properly assess risk. In particular, ethnic differences in the genetic background of thrombophilia have been reported. We recently conducted a large-scale analysis of the plasma activities of proteins C, S, antithrombin, and plasminogen within the Japanese general population. We found age- and sex-related differences and estimated the prevalence of deficiencies of protein C (0.13%), antithrombin (0.15%), protein S (1.12%), and plasminogen (4.29%). We also evaluated the genetic contribution to deep vein thrombosis and found that protein S mutation K196E is a genetic risk factor in the Japanese population. We estimated allele frequency to be 0.009, suggesting that 1 of 12,000 Japanese may be homozygous for the E allele, thus possibly as many as 10,000 individuals. Accordingly, a substantial proportion of the Japanese population carries the protein S E allele and is at risk of developing deep vein thrombosis. Given the frequency of this mutation and its strong correlation with deep vein thrombosis, it may be valuable to conduct a large-scale screening for this allele and advise concerned persons to avoid environmental risk factors known to be associated with deep vein thrombosis.

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Key words: Protein S; Deep vein thrombosis; Genetic risk; General population

1. Two Natural Anticoagulant Pathways: Protein C System and Protease Inhibitor System

Regulation of coagulation is achieved by a finely tuned balance between procoagulant and anticoagulant potencies. Generation of the multifunctional protease thrombin is a key event resulting from activation of the blood coagulation system. To regulate thrombin formation in plasma, 2 anticoagulant systems act in synergy. The first is known as the protein C anticoagulant pathway, the second as the heparan sulfate–dependent protease inhibitor system [1,2]. The protein C system controls 2 critical reactions: activation of factor X and activation of prothrombin. In this system, the thrombin-thrombomodulin complex activates protein C bound to its endothelial cell receptor, which is constitutively expressed. Resulting activated protein C (APC) has a relatively long half-life in circulation (approximately 20 minutes) and proteolytically inactivates activated factors V (FVa) and VIII (FVIIIa). Protein S accelerates inactivation of FVa and FVIIIa by APC. In the protease inhibitor system, antithrombin and tissue factor pathway inhibitor neutralize key coagulation proteases, in particular activated factors VII, IX, and X, in addition to thrombin. Inactivation of these proteases is heparan sulfate–dependent and occurs on the endothelium, lowering the potency of coagulant activity. Thus 2 systems involving a total of 6 proteins mainly control coagulation. Genetic or acquired deficiencies of any of these proteins may lead to vein thrombosis. Deficiency in protein C, protein S, or antithrombin is a major risk factor for vein thrombosis among white people [2,3]. Lack of data concerning the prevalence of these deficiencies in the general population of other ethnic groups renders it hazardous to extrapolate risk factors for vein thrombosis.

Correspondence and reprint requests: Toshiyuki Miyata, PhD, Director, Department of Etiology and Pathogenesis, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 5658565, Japan; 81-66833-5012; fax: 81-66835-1176 (e-mail: miyata@ri.ncvc.go.jp).

2. Ethnic Differences in Genetics of Thrombophilia

There is growing evidence that within different ethnic groups, mutations associated with disease arise with different frequencies, thus ethnicity-specific studies are needed to identify causative mutations and to properly assess risk [4,5]. It is now well documented that there are ethnic differences in the genetic background of thrombophilia.

Factor V Leiden is an established genetic risk factor predominantly found in white populations [3,6]. This factor historically was found in the plasma of patients with deep vein thrombosis [7]. When plasma showed reduced anticoagulant response to the addition of APC, the phenotype was called APC resistance. Genetic study of APC resistance revealed a single nucleotide mutation in the gene of coagulation factor V: G-to-A missense mutation at position 1691 of the transcript resulting in replacement of Arg506 with Gln [8]. Arg506 is a target site for APC-catalyzed inactivation of FVa. Therefore a simple explanation for the mechanisms of APC resistance is that the Arg506-to-Gln change endorses resistance against proteolysis by APC, leading to impaired down-regulation of FVa [1,3,6].

The factor V Leiden mutation has a high prevalence, between 2% and 15%, in the general white population [9]. The prevalence is as high as 60% in selected patients with vein thrombosis [9]. Overall, the factor V Leiden mutation is the most common genetic risk factor for vein thrombosis in white populations, yet mutation is absent in other populations, including Japanese [10].

Another genetic polymorphism, prothrombin G20210A, has been identified as a genetic risk factor for vein thrombosis in whites [11]. Carriers of the 20210A allele have an increased plasma level of prothrombin, which may be a risk factor for vein thrombosis [11]. This polymorphism has extreme difference in prevalence among various ethnic groups and is absent in the Japanese population [12].

3. The Suita Study: A Japanese General Population

The National Cardiovascular Center conducted the Suita Study for the purpose of identifying the most common risk factors or characteristics that contribute to cardiovascular disease in the Japanese population. A large group of participants without overt symptoms of cardiovascular disease who had not had a heart attack or stroke were observed over a long period [13,14]. The study was based on a random sampling of 14,200 Japanese residents of Suita, a city near Osaka and part of the second-largest urban area of Japan. The 14,200 residents, between 30 and 89 years of age, were arbitrarily selected from the city population registry and were stratified by sex and decennial boundary. Letters were sent to the selected residents asking them whether they would be willing to participate in this study, which was started in 1989 on a cohort basis. By February 1997, 52.7% of the selected subjects (n = 7347) had paid an initial visit to the National Cardiovascular Center. After February 1997, participants visited the National Cardiovascular Center every 2 years for regular health checkups. In addition to routine blood examinations (total cholesterol, high-density lipoprotein cholesterol, triglycerides, glucose, glycosylated hemoglobin [HbA1c], systolic, and diastolic blood pressure), a number of thrombosis-related parameters were measured, including antithrombin, anticoagulant proteins C and S, and fibrinolytic protein plasminogen [15-17]. Examination of these thrombosis-related parameters provides invaluable information concerning thrombosis and hemostasis, and it is reasonable to believe that results obtained in the Suita Study are representative of the Japanese general population.

Overall, 12 thrombosis-related parameters were examined in the Japanese population. Results concerning antithrombin, proteins C and S, tissue factor pathway inhibitor, and fibrinolytic protein plasminogen have been published [15-18]. We describe and summarize these results in view of plasma activity level and introduce the genetics of thrombosis.

4. Plasma Activity of Antithrombin, Proteins C and S, and Plasminogen in the Japanese General Population: Age- and Sex-Related Differences and Prevalence of Deficiency

It has been reported that deficiency of proteins C and S or antithrombin may affect 1.1% to 3% of patients with vein thrombosis and as many as 5% to 9% of patients with recurrent disease and juvenile patients [19,20]. However, in a study measuring the prevalence of protein C deficiency in the general population of Scotland, investigators found a prevalence of no more than 0.2% [21], and in a study in the Midwest of the United States, investigators found a prevalence of 0.3% to 0.5% [22]. The prevalence of antithrombin deficiency was reported to be 0.16% in the general population of Scotland [23]. Thus large studies have been performed in the United States and Europe. Only small-scale investigations have been conducted in the Asian population [24], and prevalence was not assessed.

The first report [25] linking plasminogen deficiency to thrombosis was followed by a number of publications on plasminogen deficiency. Subsequent studies, however, challenged the link between plasminogen deficiency and thrombosis [26,27]. Among 1192 consecutive patients with a history of venous and/or arterial thrombosis, plasminogen deficiency was not found to be a risk factor for thrombosis [27]. In a large cohort study performed in Scotland, investigators also found no such link. Twenty-eight persons with plasminogen deficiency were identified among 9611 donors, giving a prevalence of 0.29% [26]. This prevalence was not significantly different from the prevalence (0.54%) calculated from studies of thrombotic cohorts in the literature, suggesting that plasminogen deficiency can be excluded as a risk factor for thrombosis. Intrinsic limitations in these studies, however, prevented complete exclusion of plasminogen deficiency as a risk factor. For example, comparison of the frequencies among populations in relation to geographic distance has not been carefully examined.

We measured plasma levels of antithrombin, protein C, and plasminogen in 4517 persons from the Japanese general population. Antithrombin activity was measured through its heparin cofactor activity with S-2238 as a chromogenic substrate. Protein C level was measured after activation of protein C activator (Protac) with S-2366 as substrate. Plasminogen was measured with S-2251 as a chromogenic substrate

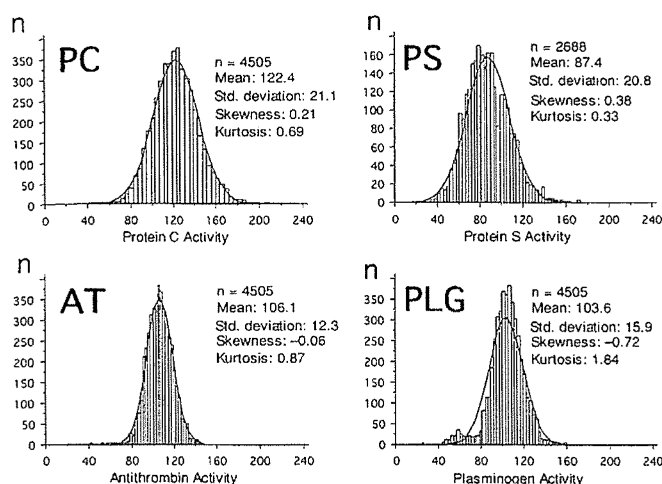

Figure 1. Distribution of plasma activity for protein C (PC), protein S (PS), antithrombin (AT), and plasminogen (PLG) in the Japanese general population. Protein C, antithrombin, and plasminogen activities were measured in 4517 subjects. Protein S activity was measured in 2690 subjects. Mean value, standard deviation, skewness, and kurtosis coefficients of each protein are shown.

after activation by streptokinase. We also used a Staclot protein S kit (Diagnostica Stago, Asniéres, France) to measure protein S cofactor efficiency in 2690 individuals in relation to the effect of APC on activated partial thromboplastin time.

Figure 1 shows distributions of proteins C and S activities as well as antithrombin and plasminogen levels in the Japanese general population. It appears that SDs for proteins C and S activity are larger than for antithrombin and plasminogen activity. Plasminogen activity is characterized by a large peak centered at 104% but also includes a small and broad peak corresponding to 60% activity. A smaller peak corresponds to plasminogen deficiency.

Figure 2 shows the age (32-89 years) and sex distribution for protein C and S activity as well as antithrombin and plasminogen levels. Analysis of activity through decennial regrouping showed that activity of all proteins was significantly reduced in men older than 50 years. A decrease in protein C and S activity was particularly noticeable. In contrast, protein C activity significantly increased in women older than 40 years. A sex-related difference also was observed for men 30 to 39 years and 40 to 49 years of age, who had higher proteins C and S activity than women in the same age groups.

Table 1 shows the mean ± SD for plasma levels of proteins C and S in 10-year age groups of men and women. In the 30- to 39-year and 40- to 49-year age groups, protein S activity was 22% and 23% lower in women than in men, respectively. In the 50- to 59-year age group, protein S activity also was 16% lower in women than in men. The reduced protein S activity in women may lead to misjudgment of protein S deficiency in women. Thus data obtained from a large general population are needed for unambiguous identification of protein S deficiency.

Sex- and age-related variation also was found with respect to antithrombin and plasminogen levels, but the differences were smaller (Figure 2). It is generally believed that thrombotic tendency in elderly persons is due to low anticoagulant activity. According to the results of our study, this finding would be true for Japanese men but not for women.

By measuring plasma activity, we estimated the prevalence of deficiency for each factor, as shown in Table 2. For protein C and antithrombin, we also calculated the ratio of antithrombin to protein C activity (AT/PC ratio). Using as the criterion an AT/PC ratio higher than 3 SD (1.27) associated with protein C activity lower than 3 SD (59.3%), we identified 6 of 4517 individuals as potentially having heterozygous protein C deficiency, implying a prevalence of 0.13%. Using the same approach and criterion, we identified 7 individuals as potentially having antithrombin deficiency, implying a prevalence of 0.15% [16]. Still using the same criterion, we identified 14 of 1252 men and 23 of 1438 women as potentially having protein S deficiency. Thus prevalence of protein S deficiency was estimated to be 1.12% in men and 1.60% in women. However, prevalence of protein S deficiency in women might have been overestimated because of interference with hormonal state. Hence we believe that 1.12% is likely to represent the true prevalence of protein S deficiency in the Japanese general population [17]. With respect to plasminogen, identification of deficiency was straightforward because of the small but distinctive peak at around 60% plasminogen activity, corresponding to individuals with plasminogen deficiency (Figure 1). To unambiguously differentiate plasminogen deficiency from normal plasminogen level, we used mean –2 SD of the calculated ratio of antithrombin to plasminogen activity (0.69) [15]. Accordingly, the prevalence of plasminogen deficiency in the Japanese population would be 4.29%.

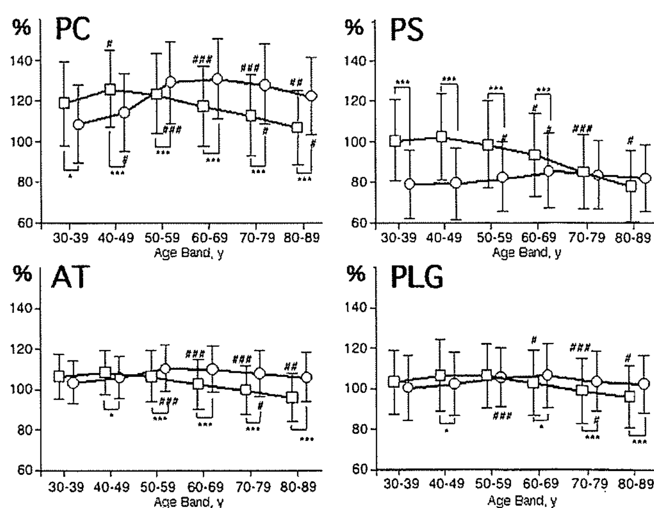

Figure 2. Sex- and age-related differences in plasma activity of protein C (PC), protein S (PS), antithrombin (AT), and plasminogen (PG) in the Japanese general population. Protein C, antithrombin, and plasminogen activity was measured in 2090 men and 2427 women. Protein S activity was measured in 1252 men and 1438 women. Activity was analyzed in 10-year age groups. Results show mean value. Error bars indicate standard deviation for each age group; squares, activity in men; circles, activity in women. #$P < .05$, ##$P < .001$, ###$P < .0001$ compared with those in the preceding age group (same sex). *$P < .05$, **$P < .001$, ***$P < .0001$ sex difference within same age group.

Table 1.
Age- and Sex-Related Distribution of Protein C and S Levels in General Population

	Protein C, %				Protein S, %			
	Men		Women		Men		Women	
Age group, y	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
30-39	66	118.9 ± 20.9	88	108.8 ± 19.2	46	100.7 ± 20.0	62	79.1 ± 17.0
40-49	262	126.0 ± 19.0	388	114.4 ± 19.3	165	102.6 ± 21.6	252	79.4 ± 17.7
50-59	373	123.8 ± 19.7	593	129.4 ± 20.2	231	98.5 ± 21.5	338	82.8 ± 17.1
60-69	660	117.8 ± 19.6	745	131.3 ± 19.8	390	93.6 ± 20.5	442	85.6 ± 18.5
70-79	555	113.1 ± 20.1	491	128.2 ± 19.7	324	85.1 ± 18.6	278	83.6 ± 17.2
80-89	167	107.1 ± 18.7	117	122.7 ± 18.9	96	78.3 ± 17.8	66	82.0 ± 16.6

Prevalence of deficiency estimated in the Japanese general population was then compared with the prevalence reported for the white general population (Table 2). The prevalence values for antithrombin and protein C deficiencies were quite similar, both conditions affecting 1 of 500 to 700 individuals. Differences were nevertheless noticeable with respect to the other factor measured. Plasminogen deficiency in particular has a high prevalence among Japanese but not among whites. Protein S deficiency may also have a higher prevalence among Japanese, even if caution is exercised about such a conclusion, because the assay used and the criteria used to define deficiency differ between studies.

We measured plasminogen, antithrombin, and protein C activity in 108 patients with deep vein thrombosis to estimate prevalence of deficiency (Table 3). Comparison of prevalence in the general population with that in the deep vein thrombosis group revealed that antithrombin and protein C deficiencies were genetic risk factors associated with deep vein thrombosis in the Japanese population (odds ratios, 38 and 52, respectively) [16]. In contrast, there was no evidence of a link between plasminogen deficiency and risk of deep vein thrombosis [15].

5. Genetic Changes in Thrombosis-Related Proteins in the Japanese Population

Factor V Leiden and prothrombin G20210A are genetic risk factors for deep vein thrombosis in white populations, but mutations have not been found in the Japanese population. No other genetic variations have been formally identified as genetic risk factors. Nevertheless, 5 genetic changes in thrombosis-related genes that may have an effect on the occurrence of deep vein thrombosis are known to be present in the Japanese population.

Table 2.
Comparison of Prevalence of Deficiencies of Proteins C and S, Antithrombin, and Plasminogen between Japanese and Westerners*

Deficiency	Population	General Population, %	DVT Group, %
Protein C	Japanese	0.13	6.5
	Westerners	0.15-0.33	3.2
Protein S	Japanese	1.12	ND
	Westerners	0.03-0.13	1.3-2.2
Antithrombin	Japanese	0.15	5.6
	Westerners	0.17	1.1
Plasminogen	Japanese	4.29	2.8
	Westerners	0.3-0.5	ND

*Data from the literature [15-17,19,21,23,53]. Prevalence in the Japanese population was estimated from the plasma activity of each factor. DVT indicates deep vein thrombosis; ND, not determined.

A missense mutation causing an Ala to Thr change at position 620 (A620T) of mature plasminogen has been identified in a Japanese patient with recurrent deep vein thrombosis [25,28]. The mutation was formerly called A601T and was referred to as plasminogen Tochigi, but the numbering standards adopted by the Nomenclature Working Group recommend that the A of the ATG of the initiator Met codon be denoted nucleotide +1 and that the initial Met residue amino acid be denoted +1 [29], causing us to rename several of the mutants we characterized. A patient with the A620T mutation exhibited decreased plasminogen activity, but antigen level was within normal limits [30]. In the mini-plasmin crystal structure, the mutation is located just before the active His residue (Ala55 in the chymotrypsin numbering system) [31]. Small-scale studies have shown that allele frequency for the plasminogen Tochigi mutation is between 0.011 and 0.021 [32,33]. The mutation has been found with an allele frequency of approximately 0.015 in the Chinese Han population and with a frequency of 0.016 in the Korean population [33] but has not been found in white populations [32].

ADAMTS13 is a von Willebrand factor (VWF)-cleaving protease [34]. Defects in the ADAMTS13 gene cause thrombotic thrombocytopenic purpura, a disease characterized by thrombocytopenia and microangiopathic hemolytic anemia with variable degrees of renal failure, neurological dysfunction, and fever. A missense mutation causing replacement of Pro475 by Ser (P475S) in the Cys-rich domain of ADAMTS13 was identified with an allele frequency of approximately 0.05 in the Japanese population [35]. Results of in vitro studies indicated the mutation has low VWF-cleaving activity. Homozygotes for this mutation retain ADAMTS13 activity and thus do not have the thrombotic thrombocytopenic purpura phenotype. Although polymorphism is found in Chinese populations at a lower frequency, it has not been identified in white populations [36-38].

Protein S is an important regulator of coagulation, and a missense mutation causing Lys196 to be replaced by Glu (K196E mutation, formerly known as protein S Tokushima and referred to as K155E mutation) within the second epidermal growth factor–like domain of protein S has been identified in Japanese patients with deep vein thrombosis [39,40]. As in the A620T mutation in plasminogen, protein S activity was decreased in carriers of the K196E mutation, but

Table 3.
Comparison of Prevalence of Protein C, Antithrombin, and Plasminogen Deficiencies between Deep Vein Thrombosis Group and General Population*

	No. of heterozygotes (prevalence)			
	Patients with DVT (n = 108)	General population (n = 4517)	Odds ratio, 95% CI (vs general population)	*P*
Protein C	7 (6.48%)	6 (0.13%)	52.1 (17.2-157.9)	<.0001
Antithrombin	6 (5.56%)	7 (0.15%)	37.9 (12.5-114.8)	<.0001
Plasminogen	3 (2.78%)	194 (4.29%)	0.6 (0.2-2.0)	.5958

*Data from Okamoto et al [15] and Sakata et al [16]. DVT indicates deep vein thrombosis; CI, confidence interval.

antigen levels were within normal limits [39,41]. Frequency of the allele has been reported to be 0.008 [39], and in vitro studies have revealed that the K196E mutation has decreased APC cofactor activity and poorly accelerated inactivation of prothrombinase [42]. Prothrombinase is the only prothrombin activator complex of the clotting cascade. It is composed of factor Xa bound to FVa on the phospholipid surface of thrombin-stimulated platelets.

A substitution in the 5′-untranslated region of the factor XII gene has been reported. It consists of a C-to-T change at nucleotide position –4 (–4C→T, formerly referred to as 46C→T) [43]. T-allele frequency is 0.73 in the Japanese population, and polymorphism is also found in white populations but with a lower frequency (0.2). Transcription of the T-allele is lower than normal, presumably because of creation of an additional ATG initiation codon and/or impairment of the consensus sequence that initiates translation according to the scanning model. Plasma level of factor XII ultimately decreases, as does, consequently, its associated activity [43]. However, the implications of this mutation in the development of thrombotic disorders is unclear [44].

A 4G/5G polymorphism within the promoter region of plasminogen activator inhibitor 1 (PAI-1) has also been reported. Individuals carrying the 4G allele exhibit higher plasma PAI-1 levels than 5G allele carriers, and the prevalence of the 4G allele appears significantly higher in patients with myocardial infarction than in population-based controls [45]. An in vitro study showed that a promoter region with the 4G allele is unable to bind a repressor protein. The consequence is that the basal level of PAI-1 transcription increases. The frequencies of 4G allele in the white and the Japanese populations have been reported to be 0.52 to 0.53 and 0.61, respectively [45-47]. As for factor XII polymorphism, the relation between the 4G allele and thrombotic disorders needs to be clarified [46].

6. Genetic Risk of Vein Thrombosis in the Japanese Population: Emerging Evidence of Protein S K196E as a Risk Factor

To address whether the 5 genetic variations described earlier are genetic risk factors for deep vein thrombosis in the Japanese population, we performed a case-control study [48]. Between December 2002 and October 2004, 161 patients with deep vein thrombosis (78 men and 83 women working under the auspices of the Ministry of Health Labor and Welfare of Japan) were registered by the Study Group of Research on Measures for Intractable Diseases. We evaluated the genetic contribution of the 5 polymorphisms by comparing their prevalence among the 161 patients who had deep vein thrombosis with their prevalence among 3655 population-based controls. Only the E allele of the protein S gene was found to be a genetic risk factor for deep vein thrombosis in the Japanese population (Table 4). No other polymorphism was found to be statistically significant between these 2 groups. Another recent study reached the same conclusion [49].

Among the population-based controls, 66 of 3651 individuals were heterozygous for the E-allele polymorphism, and none were homozygous. This finding corresponds to an allele frequency of 0.009 within the Japanese population, consistent with the previously reported frequency of 0.0082 [39]. Extrapolating from these values, we estimate that approximately 1 of every 12,000 Japanese individuals is homozygous for the E allele, representing a total of as many as 10,000 individuals. Thus a substantial number of Japanese carry the E allele for protein S and risk of development of deep vein thrombosis. Given the frequency of this mutation and its strong correlation with deep vein thrombosis, it may be advisable to screen individuals for the E allele and recommend that carriers avoid environmental risk factors associated with deep vein thrombosis.

7. Perspectives on Protein S K196E Mutation Research

Protein S K196E mutation is an established genetic risk factor for deep vein thrombosis among Japanese persons and has been confirmed in 2 independent studies [48,49]. Whether the K196E mutation is also a risk factor for arterial occlusive disease and other thrombotic diseases, such as recurrent abortion, is unknown. In the case of factor V Leiden, a possible link between mutation and arterial occlusive disease is a controversial issue. It is clear that the K196E mutation in protein S is not as dramatic as would be complete deficiency of protein S or C: no homozygote carrier was identified in infants with severe purpura fulminans. It is likely that the risk of thrombosis in individuals with K196E protein S is greatly influenced by acquired risk factors. Presence of protein S K196E in addition to other risk factors associated with thrombosis, such as pregnancy, use of oral contraceptives, trauma, and an additional defect in protein C, protein S, or antithrombin, may dramatically exacerbate thrombosis tendency. Predisposing factors may act synergistically to increase the risk of thrombosis due to protein S K196E mutation.

Protein S K196E mutation was originally identified in 1993 in Japanese patients with deep vein thrombosis and belonging to 2 independent families [39,40]. In vitro studies showed that protein S with E196 has diminished capability to act as an APC cofactor. In addition, compared with the wild type, protein S

Table 4.
Number and Genotype Frequency of 5 Polymorphisms in Deep Vein Thrombosis and Control Groups

	Plasminogen (A620T)	ADAMTS13 (P475S)	Protein S (K196E)	Factor XII (−4C→T)	Plasminogen Activator Inhibitor 1 (4G/5G)
Deep vein thrombosis					
Major homozygous	152	139	146	63	61
Heterozygous	9	20	13	75	69
Minor homozygous	0	1	2	23	30
Total	161	160	161	161	160
Minor allele frequency	0.028	0.069	0.053	0.376	0.403
General population					
Major homozygous	3501	3290	3585	1513	1468
Heterozygous	149	332	66	1651	1686
Minor homozygous	0	17	0	486	497
Total	3650	3639	3651	3650	3651
Minor allele frequency	0.020	0.050	0.009	0.359	0.367
χ^2	0.987	2.179	75.464	0.372	3.402
P	.320	.336	<.001	.830	.183

with E196 has reduced ability to inhibit the prothrombinase complex, suggesting that mutant protein S cannot interact efficiently with both APC and factor Xa [42,50]. However, protein S activity in the plasma of carriers with the mutant allele is controversial. In 1 family, carriers had protein S activity within the normal range [39], whereas in the other family protein S activity was lower than normal [41]. Although protein S K196E mutation is directly linked to development of deep vein thrombosis, measurable protein S activity is not always reduced. This finding indicates that protein S activity may not be the proper tool for detecting plasma deficiency. Therefore genetic analysis or other direct discrimination methods must be used for proper identification of protein S K196E carriers.

Overall, the protein S K196E mutation seems to occur mainly in eastern Asian populations, but its exact geographical distribution is an important issue. The A620T mutation in plasminogen occurs with an allele frequency of 0.014 to 0.015 in the Chinese Han population and with an allele frequency of 0.016 [33] in the Korean population. It is likely that the allele frequency is similar in the Japanese population. In contrast, ADAMTS13 polymorphism has an allele frequency of 0.05 in the Japanese population but is found at a lower frequency in the Chinese population [37]. After haplotype analysis, the origin of the factor V Leiden mutation was traced back 100,000 years, to a period after the out-of-Africa migration. A more recent mutation is that of the Z allele of α_1-antitrypsin, which occurred only 6000 years ago in northern Europe [51,52]. Using a similar approach, it would be interesting to trace the origin of the protein S K196E mutation, when and where it occurred first, and how it spread in the Asian population.

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References

1. Dahlback B. Progress in the understanding of the protein C anticoagulant pathway. *Int J Hematol.* 2004;79:109-116.
2. Dahlback B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. *Arterioscler Thromb Vasc Biol.* 2005;25:1311-1320.
3. Nicolaes GA, Dahlback B. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol.* 2002;22:530-538.
4. Cargill M, Altshuler D, Ireland J, et al. Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet.* 1999;22:231-238.
5. Stephens JC, Schneider JA, Tanguay DA, et al. Haplotype variation and linkage disequilibrium in 313 human genes. *Science.* 2001;293:489-493.
6. Kalafatis M, Mann KG. Factor V Leiden and thrombophilia. *Arterioscler Thromb Vasc Biol.* 1997;17:620-627.
7. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A.* 1993;90:1004-1008.
8. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature.* 1994;369:64-67.
9. Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet.* 1995;346:1133-1134.
10. Fujimura H, Kambayash J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost.* 1995;74:1381-1382.
11. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood.* 1996;88:3698-3703.
12. Miyata T, Kawasaki T, Fujimura H, Uchida K, Tsushima M, Kato H. The prothrombin gene G20210A mutation is not found among

Japanese patients with deep vein thrombosis and healthy individuals. *Blood Coagul Fibrinolysis.* 1998;9:451-452.

13. Kokubo Y, Inamoto N, Tomoike H, et al. Association of genetic polymorphisms of sodium-calcium exchanger 1 gene, NCX1, with hypertension in a Japanese general population. *Hypertens Res.* 2004;27:697-702.
14. Kamide K, Kokubo Y, Yang J, et al. Hypertension susceptibility genes on chromosome 2p24-p25 in a general Japanese population. *J Hypertens.* 2005;23:955-960.
15. Okamoto A, Sakata T, Mannami T, et al. Population-based distribution of plasminogen activity and estimated prevalence and relevance to thrombotic diseases of plasminogen deficiency in the Japanese: the Suita Study. *J Thromb Haemost.* 2003;1:2397-2403.
16. Sakata T, Okamoto A, Mannami T, Matsuo H, Miyata T. Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese. *J Thromb Haemost.* 2004;2:528-530.
17. Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. *J Thromb Haemost.* 2004;2:1012-1013.
18. Sakata T, Mannami T, Baba S, et al. Potential of free-form TFPI and PAI-1 to be useful markers of early atherosclerosis in a Japanese general population (the Suita Study): association with the intimal-medial thickness of carotid arteries. *Atherosclerosis.* 2004;176:355-360.
19. De Stefano V, Finazzi G, Mannucci PM. Inherited thrombophilia: pathogenesis, clinical syndromes, and management. *Blood.* 1996;87:3531-3544.
20. Lane DA, Mannucci PM, Bauer KA, et al. Inherited thrombophilia: part 1. *Thromb Haemost.* 1996;76:651-662.
21. Tait RC, Walker ID, Reitsma PH, et al. Prevalence of protein C deficiency in the healthy population. *Thromb Haemost.* 1995;73:87-93.
22. Miletich J, Sherman L, Broze G Jr. Absence of thrombosis in subjects with heterozygous protein C deficiency. *N Engl J Med.* 1987;317:991-996.
23. Tait RC, Walker ID, Perry DJ, et al. Prevalence of antithrombin deficiency in the healthy population. *Br J Haematol.* 1994;87:106-112.
24. Suehisa E, Nomura T, Kawasaki T, Kanakura Y. Frequency of natural coagulation inhibitor (antithrombin III, protein C and protein S) deficiencies in Japanese patients with spontaneous deep vein thrombosis. *Blood Coagul Fibrinolysis.* 2001;12:95-99.
25. Aoki N, Moroi M, Sakata Y, Yoshida N, Matsuda M. Abnormal plasminogen. A hereditary molecular abnormality found in a patient with recurrent thrombosis. *J Clin Invest.* 1978;61:1186-1195.
26. Tait RC, Walker ID, Conkie JA, Islam SI, McCall F. Isolated familial plasminogen deficiency may not be a risk factor for thrombosis. *Thromb Haemost.* 1996;76:1004-1008.
27. Demarmels Biasiutti F, Sulzer I, Stucki B, Wuillemin WA, Furlan M, Lammle B. Is plasminogen deficiency a thrombotic risk factor? A study on 23 thrombophilic patients and their family members. *Thromb Haemost.* 1998;80:167-170.
28. Miyata T, Iwanaga S, Sakata Y, Aoki N. Plasminogen Tochigi: inactive plasmin resulting from replacement of alanine-600 by threonine in the active site. *Proc Natl Acad Sci U S A.* 1982;79:6132-6136.
29. Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat.* 1998;11:1-3.
30. Sakata Y, Aoki N. Molecular abnormality of plasminogen. *J Biol Chem.* 1980;255:5442-5447.
31. Wang X, Lin X, Loy JA, Tang J, Zhang XC. Crystal structure of the catalytic domain of human plasmin complexed with streptokinase. *Science.* 1998;281:1662-1665.
32. Aoki N, Tateno K, Sakata Y. Differences of frequency distributions of plasminogen phenotypes between Japanese and American populations: new methods for the detection of plasminogen variants. *Biochem Genet.* 1984;22:871-881.
33. Ooe A, Kida M, Yamazaki T, et al. Common mutation of plasminogen detected in three Asian populations by an amplification refractory mutation system and rapid automated capillary electrophoresis. *Thromb Haemost.* 1999;82:1342-1346.
34. Sadler JE, Moake JL, Miyata T, George JN. Recent advances in thrombotic thrombocytopenic purpura. *Hematology (Am Soc Hematol Educ Program).* 2004;407-423.
35. Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor–cleaving protease activity. *Proc Natl Acad Sci U S A.* 2002;99:11902-11907.
36. Kokame K, Miyata T. Genetic defects leading to hereditary thrombotic thrombocytopenic purpura. *Semin Hematol.* 2004;41:34-40.
37. Ruan C, Dai L, Su J, Wang Z, Ruan C. The frequency of P475S polymorphism in von Willebrand factor–cleaving protease in the Chinese population and its relevance to arterial thrombotic disorders. *Thromb Haemost.* 2004;91:1257-1258.
38. Bongers TN, De Maat MP, Dippel DW, Uitterlinden AG, Leebeek FW. Absence of Pro475Ser polymorphism in ADAMTS-13 in Caucasians. *J Thromb Haemost.* 2005;3:805.
39. Yamazaki T, Sugiura I, Matsushita T, et al. A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. *Thromb Res.* 1993;70:395-403.
40. Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor–like domain of protein S. *Blood.* 1994;83:683-690.
41. Shigekiyo T, Uno Y, Kawauchi S, et al. Protein S Tokushima: an abnormal protein S found in a Japanese family with thrombosis. *Thromb Haemost.* 1993;70:244-246.
42. Hayashi T, Nishioka J, Suzuki K. Characterization of dysfunctional protein S-Tokushima (K155→ E) in relation to the molecular interactions required for the regulation of blood coagulation. *Pol J Pharmacol.* 1996;48:221-223.
43. Kanaji T, Okamura T, Osaki K, et al. A common genetic polymorphism (46 C to T substitution) in the 5′-untranslated region of the coagulation factor XII gene is associated with low translation efficiency and decrease in plasma factor XII level. *Blood.* 1998;91:2010-2014.
44. Bertina RM, Poort SR, Vos HL, Rosendaal FR. The 46C→ T polymorphism in the factor XII gene (F12) and the risk of venous thrombosis. *J Thromb Haemost.* 2005;3:597-599.
45. Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci U S A.* 1995;92:1851-1855.
46. Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Miletich JP. Arterial and venous thrombosis is not associated with the 4G/5G polymorphism in the promoter of the plasminogen activator inhibitor gene in a large cohort of US men. *Circulation.* 1997;95:59-62.
47. Matsubara Y, Murata M, Isshiki I, et al. Genotype frequency of plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism in healthy Japanese males and its relation to PAI-1 levels. *Int J Hematol.* 1999;69:43-47.
48. Kimura R, Honda S, Kawasaki T, et al. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood.* 2006;107:1737-1738.
49. Kinoshita S, Iida H, Inoue S, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem.* 2005;38:908-915.
50. Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S(Tokushima) (Lys155→ Glu) for the regulation of the blood coagulation system. *Biochim Biophys Acta.* 1995;1272:159-167.
51. Cox DW, Woo SL, Mansfield T. DNA restriction fragments associated with alpha 1-antitrypsin indicate a single origin for deficiency allele PI Z. *Nature.* 1985;316:79-81.
52. Zivelin A, Griffin JH, Xu X, et al. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood.* 1997;89:397-402.
53. Dykes AC, Walker ID, McMahon AD, Islam SI, Tait RC. A study of protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. *Br J Haematol.* 2001;113:636-641.

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Polymorphisms in Vitamin K–Dependent γ-Carboxylation–Related Genes Influence Interindividual Variability in Plasma Protein C and Protein S Activities in the General Population

Rina Kimura,[a] Yoshihiro Kokubo,[b] Kotaro Miyashita,[c] Ryoichi Otsubo,[c] Kazuyuki Nagatsuka,[c] Toshiho Otsuki,[c] Toshiyuki Sakata,[d] Junko Nagura,[b] Akira Okayama,[b] Kazuo Minematsu,[c] Hiroaki Naritomi,[c] Shigenori Honda,[a] Kiyoshi Sato,[d] Hitonobu Tomoike,[b] Toshiyuki Miyata[a]

[a]Research Institute, [b]Department of Preventive Cardiology, [c]Cerebrovascular Division, Department of Medicine, and [d]Laboratory of Clinical Chemistry, National Cardiovascular Center, Suita, Osaka, Japan

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Abstract

γ-Glutamyl carboxylation, a reaction essential for the activity of vitamin K–dependent proteins, requires the concerted actions of γ-glutamyl carboxylase (GGCX), vitamin K 2,3-epoxide reductase complex 1 (VKORC1), and the chaperone calumenin (CALU). We evaluated the contribution of genetic polymorphisms in *VKORC1*, *GGCX*, and *CALU* to interindividual variation in the activities of plasma protein C and protein S. We sequenced these 3 genes in 96 Japanese individuals and genotyped 9 representative single-nucleotide polymorphisms in 3655 Japanese individuals representative of the general population. The mean activity of protein C in women bearing the GG genotype of *GGCX* 8016G>A (130.8% ± 1.5%, n = 156) was significantly greater (P = .002) than that of individuals with either the AG (126.8% ± 0.7%, n = 728) or the AA (125.4% ± 0.6%, n = 881) genotype, after adjusting for confounding factors. The *GGCX* 8016G>A change leads to the substitution of Gln for Arg at amino acid residue 325 (Arg325Gln). This effect was comparable to that of a previously defined polymorphism in the protein C promoter. Mean protein S activity was influenced by the *VKORC1* 3730G>A and *CALU* 20943T>A genotypes, after adjusting for confounding factors. Thus, polymorphisms in genes involved in the vitamin K–dependent γ-carboxylation reaction influence interindividual variation in the activities of protein C and protein S in the general population.
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Key words: Genetic polymorphism; Vitamin K; Protein C activity; γ-Glutamyl carboxylase; Vitamin K epoxide reductase complex subunit 1; Calumenin

1. Introduction

Some proteins require the vitamin K–dependent post-translational modification of specific glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla) residues for proper functioning [1]; such vitamin K–dependent proteins include blood coagulation factors (prothrombin, factors VII, IX, and X, and proteins S, C, and Z) and proteins involved in calcium homeostasis (osteocalcin and matrix Gla protein), cell growth (Gas6), and signal transduction (PRGP1 and PRGP2) [2]. This γ-carboxyl modification enables calcium binding, which is essential for the physiological action of these proteins [3].

γ-Glutamyl carboxylase (GGCX) and the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR) mediate vitamin K–dependent γ-carboxylation in the endoplasmic reticulum (ER) [4]. GGCX is a 94-kd, ER-resident protein with 5 transmembrane domains [5,6]. Vitamin K epoxide reductase complex subunit 1 (VKORC1) is an 18-kd ER protein with 3 transmembrane domains containing a thioredoxin-like CXXC redox center [7-11]; this protein is thought to be a subunit of the VKOR enzyme complex. VKORC1 activity is the rate-limiting step in the γ-carboxylation reaction [10,12]. GGCX catalyzes the addition of carbon dioxide

Correspondence and reprint requests: Toshiyuki Miyata, PhD, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan; 81-6-6833-5012 ext 2512; fax: 81-6-6835-1176 (e-mail: miyata@ri.ncvc.go.jp).

to Glu to form Gla. This reaction requires reduced vitamin K as a cofactor and generates oxidized vitamin K 2,3-epoxide. VKOR reduces vitamin K 2,3-epoxide back to the reduced form of vitamin K; this cyclic conversion of vitamin K metabolites is called the vitamin K cycle.

VKORC1 is a target enzyme of warfarin. Warfarin interferes with the formation of Gla residues by inhibiting VKOR activity, ultimately resulting in reduced levels of vitamin K–dependent coagulation factors. Recent studies have shown that genetic polymorphisms in *VKORC1* explain the interindividual variability in the warfarin maintenance dose [13-19]. The allele frequencies of the polymorphisms in *VKORC1* differ widely among ethnic groups, and such variation in part explains why people from Asian populations require a much lower maintenance dose of warfarin than Caucasians [11,14,16,20,21].

The ER-resident protein calumenin (CALU), a soluble protein with an ER-retention signal at its C terminus, has an EF-hand structure typical of Ca^{2+}-binding proteins [22]. A recent study suggested that CALU inhibits the γ-carboxylation reaction [23]. Small interfering RNA–mediated CALU knock-down caused a 5-fold increase in γ-carboxylase activity, suggesting a regulatory role for this protein in the biosynthesis of functional vitamin K–dependent proteins [24].

Protein C and protein S are anticoagulant proteins with Gla domains at their N termini. On the endothelium surface, protein C binds the protein C receptor and is activated by the thrombin-thrombomodulin complex. The resulting activated protein C associates with protein S to catalyze the proteolytic inactivation of factors Va and VIIIa [25,26]. Ca^{2+} binding to the Gla domain of protein C and protein S is essential for their anticoagulant activities.

We hypothesized that polymorphisms in genes controlling γ-carboxylation could affect plasma levels of vitamin K–dependent anticoagulant proteins. Therefore, we sequenced *GGCX*, *VKORC1*, and *CALU* to identify single-nucleotide polymorphisms (SNPs) that correlated with levels of protein C and protein S in the Japanese population. Additionally, we examined the relationship between protein C activity and SNPs in the protein C gene (*PROC*) promoter (−1657C>T and −1644G>A) and the NAD(P)H:dehydrogenase quinone 1 gene (*NQO1* 2515C>T), which have previously been linked with protein C levels [27-29].

2. Methods

2.1. Study Individuals

We screened 96 unrelated Japanese stroke patients admitted to the Cerebrovascular Division of the National Cardiovascular Center between November 2003 and March 2004 for genetic variation. For the sample from the general population, we randomly selected Japanese volunteers from Suita city residents, compiled as part of the previously described Suita Study [30]. In that study, individuals visited the National Cardiovascular Center every 2 years for regular health checkups. Diabetes mellitus was defined as a fasting blood glucose level ≥126 mg/dL or current use of insulin or oral antidiabetic agents. Hypertension was diagnosed by a systolic blood pressure ≥140 mm Hg, a diastolic blood pressure ≥90 mm Hg, or current use of antihypertensive medications. Hyperlipidemia was defined as a total cholesterol level ≥220 mg/dL or current use of antihyperlipidemia medications at the time of the examination. Body mass indices were calculated as the weight in kilograms divided by the height in meters squared. Leukocyte DNA was collected from participants between April 2002 and February 2004 [31]. We determined the genotypes of 3655 participants (1709 men and 1946 women). All participants gave their written informed consent for genetic analysis. This study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

2.2. Coagulation Assays

Blood samples for coagulation assays were collected after the subject had fasted overnight. Protein C amidolytic activity was measured with the chromogenic substrate S-2366 (KabiVitrum, Stockholm, Sweden) and Protac (Pentapharm, Basel, Switzerland) as the activator [32]. The cofactor activity for activated protein C was used to measure protein S activity with an activated partial thromboplastin time assay (Staclot Protein S; Diagnostica Stago, Asnières, France) [33]. The activities of protein C and protein S were expressed as a percentage of the activity in control plasma. The intra-assay coefficient of variation was 1.3% (n = 10) for protein C, and 6.9% (n = 10) for protein S. Protein C and protein S activities were determined for 3295 and 1866 individuals, respectively, in the general population.

2.3. Genetic Analyses

Direct sequencing was performed as described [34]. Genetic variations were identified with NAMIHEI (version 1.0; Mitsui Knowledge Industry, Tokyo, Japan) and Sequencher (version 4.0; Gene Codes, Ann Arbor, MI, USA) software, followed by visual inspection. We have adopted the numbering standards of the Nomenclature Working Group, in which the A of the initiation Met codon (ATG) is denoted as nucleotide +1 and the initial Met residue is denoted as amino acid residue +1 [35]. Genotyping of SNPs was performed by TaqMan–polymerase chain reaction (PCR) analysis (Applied Biosystems, Foster City, CA, USA), as previously described [36]. The PCR primers used for direct sequencing and the probes and conditions for the TaqMan-PCR analysis are available upon request.

2.4. Statistical Analyses

The Hardy-Weinberg equilibrium was assessed by the χ^2 test. Pair-wise linkage disequilibrium between 2 polymorphisms was evaluated by determining r^2 with SNPAlyze software (version 3.1; Dynacom, Kanagawa, Japan). Associations between genotypes and either plasma protein C activity or protein S activity were examined for each sex by 1-way analysis of variance without adjustment or by multiple regression analyses with adjustment for confounding variables, including age, body mass index, smoking, current alcohol consumption, and the presence of diabetes mellitus, hypertension, and hyperlipidemia. Statistical analyses were

Table 1.
VKORC1, GGCX, and CALU Polymorphisms and Allele Frequencies Identified by Direct Sequencing of Samples from 96 Japanese Individuals*

Gene	SNPs†	LD‡	Amino Acid Change	Region	Allele Frequency		Flanking Sequence	Genotyping§	rs ID No.
					Allele 1	Allele 2			
VKORC1	184G>T			Intron 1	0.989	0.011	tctcctccaggtgtgcacgg[G/T] agtgggaggcgtggggcctc		
	403G>T	A		Intron 1	0.995	0.005	attattccggggactcgcac[G/T] tgaattggatgccaaggaat		
	523G>A			Intron 1	0.968	0.032	ggggtgttggcgatggctgc[G/A] cccaggaacaaggtggcccg	Done	
	859A>T			Intron 1	0.995	0.005	ctaccaaaaatagaaaaatt[A/T] atcgggcgctatggcgggtg		
	1173C>T	B		Intron 1	0.036	0.966	ggtgccaggagatcatcgac[C/T] cttggactaggatgggaggt		rs9934438
	1338A>G		His68Arg	Exon 2	0.995	0.005	gggtttcgggctggtggagc[A/G] tgtgctgggacaggacagca	Done	
	1411A>G	A	Leu92	Exon 2	0.995	0.005	atcttctacacactacagct[A/G] ttgttaggtgagtggctccg		
	1446insGCCCC			Intron 2	0.995	0.005	ctccgccccctccctgccc[-----/ Gcccc]gcccgccccgcccctcatcc		
	1542G>C	B		Intron 2	0.046	0.954	gccagctagctgctcatcac[G/C] gagcgtcctgcgggtgggga		rs8050894
	2255T>C	B		Intron 2	0.957	0.043	ctgagggaggcccagcacca[T/C] ggtcctggctgacacatggt		rs2359612
	3730G>A	B		Exon 3	0.958	0.042	cccctcctcctgccataccc[G/A] cacatgacaatggaccaaat	Done	rs7294
	3839G>A			Exon 3	0.990	0.010	ctctttccattgcccaggga[G/A] ggaaggttctgagcaataaa		
GGCX	–310A>C			Promoter	0.995	0.005	aaggaagttggggaactcag[A/C] ctcttttcagtgtttaaaaa		
	–222G>T			Promoter	0.995	0.005	tggagatcaacggcactaag[G/T] ggtacctttgccccgctcca		
	–187G>T			5′ UTR	0.989	0.011	gctccaaggctccttgttcc[G/T] ccccctgccgccaagcaagg		
	–101G>A			5′ UTR	0.989	0.011	gctttgcggccggccgcaca[G/A] ccgctgacgcgtcgggaggc		
	–70G>A	C		5′ UTR	0.995	0.005	gtcgggaggCggagcctagg[G/A] aagcaaattctcctggcggc		
	15C>T		Ala5	Exon 1	0.995	0.005	agagcaatggcggtgtctgc[C/T] gggtccgcgcggacctcgcc		
	150delT	D		Intron 1	0.646	0.354	ccggtgaggcggggggggg[T/-] cctctgtggggaagggggcg		rs11350741
	282G>T	D		Intron 1	0.634	0.366	gacattaggggatcgtggag[G/T] ggggctgcactgcagggatg		rs10172544
	307C>T			Intron 1	0.995	0.005	ctgcactgcagggatgtttc[C/T] tatagtcagatcattgaggc		
	377A>T	D		Intron 1	0.634	0.366	aagctctgagctgttggtgc[A/T] gtgatttctttgatttgagg		rs7568458
	412G>A			Intron 1	0.860	0.140	ttgaggtggagcccaccccc[G/A] cacacatattttctccattt	Done	rs1254898
	2478C>T	C	Asp113	Exon 3	0.995	0.005	ctacgcccactgccacttga[C/T] tggatgtatcttgtctacac		rs6751560
	5165G>A			Intron 4	0.995	0.005	caatgtttacctccctcggt[G/A] taggtctgtggacggtctgc		
	5424A>C	D		Intron 5	0.630	0.370	aatcccttaatctctgaatt[A/C] tcttatttgtcaaggaaaga		rs6738645
	5697A>G			Intron 5	0.989	0.011	ataagtagattatttgggtc[A/G] aggactgtaatatttgggtc		
	6146A>G			Intron 6	0.995	0.005	gctcttgggagatatatggt[A/G] tagataggcaagaaaggaaa		
	6972(CAA)$_{8\text{-}12}$			Intron 6	—	—	gacagagtgaaacttcgttt[(CAA)$_{8\text{-}12}$] acaacctagaggagtgtctt		rs10654848

Continued

Table 1
Continued

	8016G>A	D	Arg325Gln	Exon 8	0.693	0.307	gctggtgtcctactgcccccc[G/A] aaggttgcaacaactgttgc	Done	rs699664
	8021T>C	D	Leu327	Exon 8	0.307	0.693	tgtcctactgcccccgaagg[T/C] tgcaacaactgttgcccctc		
	8445C>T		Thr414	Exon 9	0.938	0.063	caccagcacgtgaagatcac[C/T] taccgtgatggccgcactgg	Done	rs10179904
	8838G>A			Intron 9	0.995	0.005	agttgataatgatgaggtgg[G/A] actaatatgggggtttgggg		
	9078C>T			Intron 10	0.995	0.005	tttttaccaagatgaacagc[C/T] cttgcttcctggtattgtct		
	9342-9344delCTT	D		Intron 10	0.741	0.259	catgctgacacaggccattt[Ctt/---] ctattccctgtccttaggaa		
	9654C>T			Intron 11	0.995	0.005	ggagaggcttttctgtcttt[C/T] cttctggcttccttggccag		
	9980-9981insA	C		Intron 11	0.995	0.005	gggattaacaacagcagaaa[-/A] gaactgagtgcagtagctca		
	10911G>A			Intron 14	0.989	0.011	atttgtcattgccatcatat[G/A] ttggcaagcttggtaacttt		
	11609-11610insG			3′ UTR	0.984	0.016	tttttttttttttggggggg[-/G] cggggttctaaagctgttt		
	12062-12063 insTAAA	D		3′ UTR	0.630	0.370	tctcaaaataaataaataaa[----/ taaa]gtggctcttggggaaaagcaa		rs5832649
	12105C>G	C		3′ UTR	0.995	0.005	taatgtaccacgatgaatag[C/G] taactgttcccaagtgtttg		
	12175G>C			3′ UTR	0.989	0.011	tacctgcattattacattag[G/C] ctgagaggtaaaataatttg		
CALU	−9755G>T	E		Promoter	0.978	0.022	ccagagcgcaagatgaggggg[G/T] agtgttgggagatggagcta		
	−9459$(GGGGCT)_{3-7}$			Promoter	—	—	gcccacgccacccgcagtac[$(GGGGCT)_{3-7}$] ggagcccgccgtcccaacaa		
	−9429G>T	E		Promoter	0.979	0.021	gtcccaacaatcccggggac[G/T] agagttgggcgggaatccgc		rs7795071
	−9171T>C			5′ UTR	0.974	0.026	gcggccacggcatcctgtgc[T/C] gtgggggctacgaggaaagg		
	−97G>A	F		Intron 1	0.927	0.073	atatgtatatcaaattaata[G/A] tttaactcggatgtgagctt		
	11G>A		Arg4Gln	Exon 2	0.792	0.208	tctaattatcatggacctgc[G/A] acagtttcttatgtgcctgt	Done	rs2290228
	344G>A			Intron 2	0.885	0.115	tattaatacatcttctgaaa[G/A] ctaaccatggaattactacc	Done	rs2290227
	5969C>T	F		Intron 3	0.922	0.078	gattgtagataaaatagacg[C/T] ggataaagatgggtttgtga		rs2307040
	5970G>A	G		Intron 3	0.990	0.010	attgtagataaaatagacgc[G/A] gataaagatgggtttgtgac		
	10195G>T			Intron 3	0.995	0.005	aattatttcttccttgcatg[G/T] ttttatctttattggaaatt		
	10587A>G	H		Intron 4	0.990	0.010	catatcactgtatatgctat[A/G] taagtattggccagattcag		rs339097
	10594T>C	F		Intron 4	0.932	0.068	ctgtatatgctatataagta[T/C] tggccagattcagaatccaa		
	11484G>A			Intron 5	0.990	0.010	aattagttgggtgtggtggc[G/A] ggcgcctgtaatcccagcta		
	18631A>G	H		Intron 5	0.989	0.011	atttctgcatttattaaggt[A/G] taagaactctttgctctcat		
	18642-18643delTT			Intron 5	0.995	0.005	tattaaggtataagaactct[TT/--] gctctcatgtgacttaaaa		
	18776A>T			Intron 5	0.995	0.005	gtagctgctgttgggttcca[A/T] acaaatggatgaggaaaaag		
	20588A>G	F		3′ UTR	0.932	0.068	ggcatgatgagttctgagct[A/G] cggaggaaccctcatttcct		rs1043550
	20674C>T	I		3′ UTR	0.995	0.005	ttgcgctactgagactgtta[C/T] tacaaactttttaagacatg		

Continued

Table 1
Continued

20943T>A	F	3′ UTR	0.927	0.073	ttactctttctcaacccctt[T/A] tatgattttaataattctca	Done	rs11653
21375G>A	F	3′ UTR	0.927	0.073	aaactaaaggaaaaatacaa[G/A]tgttttcggggcatacattt		rs1043595
22706-22707insT	G	3′ UTR	0.990	0.010	ccctttgaaattttttttttt[-/T] gtttgtttaaatcaagc		
22983C>T	F	3′ Flanking	0.926	0.074	gtcagaatgggaactctcct[C/T] gaagttctcccaaactcaga		rs1044226
23087C>T	F	3′ Flanking	0.937	0.063	ccttctcaccacagccataa[C/T] cctttttacttccattagg		
23194G>A		3′ Flanking	0.005	0.995	atgtacacacacgtttatct[G/A] tagagcataaggtaaaacaa		
23340A>T	I	3′ Flanking	0.995	0.005	taagacaaatataaaggttt[A/T] gccaaatgcaacggggagga		

*A of the initiation Met codon (ATG) is denoted as nucleotide +1, as recommended by the Nomenclature Working Group [35]. The nucleotide numbers for *VKORC1*, *GGCX*, and *CALU* are according to the reference sequences of GenBank accession IDs NC_000016.7 (region, 31137750..31142749), NC_000002.8 (region, 85750000..85788000), and NC_000007.10 (region, 127932398..127966000). SNP indicates single-nucleotide polymorphism; LD, linkage disequilibrium; UTR, untranslated region.

†SNPs are expressed as the nucleotide position of the polymorphism and the nature of the polymorphism. G>T indicates G in allele 1 and T in allele 2; ins, insertion; del, deletion.

‡The apparent LD, defined as $r^2 > 0.5$, is indicated by a letter.

§SNPs for large-scale genotyping.

performed with JMP (version 5.1) and SAS (version 6.0) software packages (SAS Institute, Cary, NC, USA).

3. Results

3.1. Identification of Genetic Variation in VKORC1, GGCX, and CALU

We sequenced the entire 5.3-kb region of *VKORC1*, including all exons and introns, and approximately 1.0 kb of the promoter region. For *GGCX* and *CALU*, we also sequenced all exons and flanking regions and sequenced approximately 0.9 kb of the promoter regions. We identified genetic variation in these genes in 96 individuals (Table 1). Eleven SNPs and 1 insertion/deletion polymorphism were identified within the *VKORC1* gene, and the allelic frequencies of all identified SNPs were <0.05, indicating that these were rare genetic variations. One individual was heterozygous for a missense mutation in the *VKORC1* gene, 1338A>G (His68Arg). In the *GGCX* gene, 27 SNPs, 3 insertion/deletions, and 1 repeat polymorphism were identified. A missense mutation, *GGCX* 8016G>A (Arg325Gln), was identified in 43 heterozygous individuals, and 8 individuals were homozygous for this variation. Finally, 23 SNPs, 1 insertion/deletion, and 1 repeat polymorphism were identified in the *CALU* gene. We identified 32 heterozygotes and 4 individuals homozygous for a missense mutation, 11G>A (Arg4Gln).

We selected SNPs with minor allele frequencies of greater than 3% for further large-scale genotyping (Table 1). We also included 1 missense mutation, *VKORC1* 1338A>G (His68Arg) with a minor allele frequency of less than 3% (Table 1), and genotyped representative SNPs when we observed linkage disequilibrium ($r^2 > 0.5$). In total, 9 SNPs in the population-based sample of 3655 individuals were selected for genotyping.

3.2. Characteristics of Population-Based Individuals

We determined protein C and protein S activities in 3295 and 1866 individuals, respectively, from the general population. The characteristics of the individuals genotyped in this study, subdivided into men (n = 1709) and women (n = 1946), are detailed in Table 2. Values for age, body mass index, smoking and drinking status, presence of diabetes mellitus or hypertension, prothrombin time, and protein S activity were significantly greater in the men. In contrast, values for hyperlipidemia and protein C activity were significantly greater in the women. Therefore, we performed the following statistical analyses after dividing the groups on the basis of sex.

3.3. Association of 9 SNPs with Protein C and Protein S Activities by 1-Way Analysis of Variance

We first analyzed the relationship between 9 SNPs and coagulation activity in 3655 Japanese individuals. The frequencies of SNPs were consistent with the expected Hardy-Weinberg equilibrium ($P > .05$), and we examined the possible association of each SNP with protein C or protein S activity by using a 1-way analysis of variance without adjustment.

Women with differences in the *GGCX* 8016G>A polymorphism exhibited significant differences in protein C activity ($P = .016$). The protein C activity (mean ± SEM) in women was 125.5% ± 0.7% (n = 881) with the GG genotype, 126.7% ± 0.8% (n = 728) with the GA genotype, and 130.6% ± 1.7% (n = 156) with the AA genotype, but the individuals with these genotypes did not differ with respect to any other identified characteristics.

Additionally, we observed significant differences in protein S activity in women with the *VKORC1* 3730G>A ($P = .033$) and *CALU* 20943T>A ($P = .028$) polymorphisms. The protein S activity (mean ± SEM) was 83.6% ± 0.6% (n = 827)

in women with the *VKORC1* 3730GG genotype, 81.6% ± 1.4% (n = 164) for those with the GA genotype, and 68.3% ± 6.6% (n = 7) for women with the AA genotype, whereas these values were 83.7% ± 0.6% (n = 873) in women with the *CALU* 20943TT genotype, 79.2% ± 1.6% (n = 123) for the TA genotype, and 82.5% ± 12.4% (n = 2) for the AA genotype. No significant differences in protein C or protein S activity were seen in men.

3.4. Multiple Logistic Analyses for SNPs Associated with Protein C and Protein S Activities

We observed significant differences in protein C or protein S activity of women with 3 SNPs examined by univariate analysis. We wished to refine these data further and therefore conducted multiple logistic analysis to eliminate sources of potentially confounding variation, including age, body mass index, percentages of smokers and alcohol consumers, and percentages of individuals with diabetes mellitus, hypertension, and hyperlipidemia.

This analysis revealed a significant association between protein C activity and *GGCX* 8016G>A (Arg325Gln) genotype in women, but not in men (Table 3). Protein C activity was significantly higher (P = .002) in women with the AA genotype (130.8% ± 1.5% [mean ± SEM], n = 156) than in women with the GA genotype (126.8% ± 0.7%, n = 728) or the GG genotype (125.4% ± 0.6%, n = 881). This association corresponded to a difference of 5.4% in protein C activity between women with the GG and AA genotypes. Women with the AA genotype (84.9% ± 1.9% [mean ± SEM], n = 80) in *GGCX* 8016G>A (Arg325Gln) tended to exhibit higher protein S activities than those with either the GA genotype (83.7% ± 0.8%, n = 418) or the GG genotype (82.4% ± 0.8%, n = 500), although these differences were not statistically significant (P = .142, Table 4).

Additionally, after we adjusted for confounding variables, women with the *VKORC1* 3730GG genotype had a significantly higher (P = .023) protein S activity (83.6% ± 0.6%, n = 827) than women with the GA genotype (81.3% ± 1.1%, n = 164) or the AA genotype (69.2% ± 6.5%, n = 7); this result was also observed in the entire population (P = .016, Table 4). Finally, women with the *CALU* 20943TT genotype had a significantly (P = .006) higher protein S activity (83.7% ± 0.6%, n = 873) than women with either the TA genotype (79.0% ± 1.6%, n = 123) or the AA genotype (82.7% ± 12.1%, n = 2). However, no significant differences in protein S activity were seen in men with different *CALU* 20943T>A genotypes (Table 4).

3.5. Comparable Effects of the GGCX 8016G>A Polymorphism to Polymorphisms in the PROC Promoter

We observed a 5.4% difference in protein C activity that depended on the genotype of the *GGCX* 8016G>A SNP (Table 3). Previous studies have linked a haplotype composed of 2 SNPs in the promoter region of *PROC*, –1657C>T (designated as –1654C>T in the literature) and –1644G>A (designated as –1641A>G), to variations in protein C activity and antigen levels in healthy subjects, as well as to the occurrence of deep vein thrombosis [27,28]. In addition, the *NQO1* 2515C>T polymorphism is associated with differences in protein C antigen levels [29]. We next wished to compare the effect of these SNPs on protein C activity in individuals with the newly identified *GGCX* 8016G>A polymorphism. Accordingly, we genotyped the 3655 Japanese individuals in our study population for these 3 SNPs and examined the results for an association between SNP genotype and protein C activity.

Table 2.
Subject Characteristics*

	Men (n = 1709)	Women (n = 1946)	P
Age, y	66.1 ± 11.3	63.5 ± 11.1	<.0001
Body mass index, kg/m^2	23.3 ± 3.0	22.4 ± 3.2	<.0001
Smoker, %	34.3	6.8	<.0001
Alcohol consumer, %	69.0	28.5	<.0001
Diabetes mellitus, %	13.0	6.1	<.0001
Hypertension, %	39.0	31.0	<.0001
Hyperlipidemia, %	29.7	47.4	<.0001
Prothrombin time, INR	0.90 ± 0.10	0.90 ± 0.07	.0232
Protein C activity, %†	119 ± 20	126 ± 21	<.0001
Protein S activity, %‡	92.8 ± 21.2	83.2 ± 17.6	<.0001

*Age, body mass index, prothrombin time, and protein C and protein S activities are presented as the mean ± SD. Diabetes mellitus was indicated by a fasting plasma glucose level ≥126 mg/dL or antidiabetic medication, hypertension was indicated by a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg or antihypertensive medication, and hyperlipidemia was indicated by a total cholesterol level ≥220 mg/dL or antihyperlipidemia medication. INR indicates international normalized ratio.

†Data are expressed as a percentage of the activity in control plasma. For men, n = 1528; for women, n = 1767.

‡Data are expressed as a percentage of the activity in control plasma. For men, n = 866; for women, n = 1000.

In our study population, neither the *PROC* –1657C>T SNP nor the *NQO1* 2515C>T SNP was associated with any differences in protein C activity, but the *PROC* –1644G>A SNP was significantly associated with variation in protein C activity (P < .001, Table 5). In individuals with the GG genotype, the protein C activity (mean ± SEM) was 119.1% ± 3.1% (n = 38), whereas it was 120.0% ± 0.7% (n = 661) for individuals with the GA genotype and 123.4% ± 0.4% (n = 2577) for individuals with genotype AA. This result corresponded to a difference of 4.3% in protein C activity between individuals with the GG and AA genotypes.

To further clarify the relationship between protein C activity and different clinical characteristics, we performed multivariate regression analysis with age, body mass index, smoking, alcohol consumption, diabetes mellitus, hypertension, hyperlipidemia, and genetic variation as variables. Protein C activity was independently associated with age, body mass index, alcohol consumption, and hyperlipidemia in women (Table 6). Additionally, 2 SNPs, *GGCX* 8016G>A and *PROC* –1644G>A, were significantly associated with differences in protein C activity, with coefficients of 4.46 (P = .006) and 3.80 (P = .001), respectively. The R^2 values for protein C activity in the model including *GGCX* 8016G>A and the model including *PROC* –1644G>A were 0.151 and 0.152,

Table 3.
Protein C Activity by *GGCX*, *VKORC1*, and *CALU* Genotype*

			Men			Women			Total		
Gene	SNP	Genotype	n	Protein C Activity, %	*P*	n	Protein C Activity, %	*P*	n	Protein C Activity, %	*P*
VKORC1	523G>A	GG	1454	118.7 ± 0.5		1672	126.4 ± 0.5		3126	122.6 ± 0.3	
		GA	72	117.1 ± 2.2		93	127.7 ± 2.0		165	122.5 ± 1.5	
		AA	0	—		0	—		0	—	
					.454			.526			.914
	1338A>G (His68Arg)	AA	1513	118.6 ± 0.5		1748	126.5 ± 0.4		3261	122.6 ± 0.3	
		AG	11	128.9 ± 5.6		13	128.4 ± 5.2		24	128.5 ± 3.9	
		GG	0	—		0	—		0	—	
					.067			.714			.129
	3730G>A	GG	1302	118.4 ± 0.5		1451	126.7 ± 0.5		2753	122.6 ± 0.4	
		GA	216	120.2 ± 1.3		302	125.7 ± 1.1		518	122.9 ± 0.8	
		AA	9	115.2 ± 6.2		12	122.1 ± 5.4		21	118.9 ± 4.1	
					.317			.292			.971
GGCX	412G>A	GG	1138	118.6 ± 0.5		1295	126.5 ± 0.5		2433	122.5 ± 0.4	
		GA	365	119.0 ± 1.0		441	126.5 ± 0.9		806	123.0 ± 0.7	
		AA	23	114.8 ± 3.9		29	126.0 ± 3.5		52	120.6 ± 2.6	
					.932			.996			.882
	8016G>A (Arg325Gln)	GG	730	118.7 ± 0.7		881	125.4 ± 0.6		1611	122.1 ± 0.5	
		GA	662	118.7 ± 0.7		728	126.8 ± 0.7		1390	122.9 ± 0.5	
		AA	135	117.8 ± 1.6		156	130.8 ± 1.5		291	124.2 ± 1.1	
					.715			.002			.069
	8445C>T	CC	1360	118.8 ± 0.5		1578	126.2 ± 0.5		2938	122.5 ± 0.4	
		CT	162	117.3 ± 1.5		177	128.4 ± 1.4		339	123.2 ± 1.0	
		TT	5	125.1 ± 8.3		10	130.8 ± 5.9		15	128.8 ± 4.9	
					.537			.100			.301
CALU	11G>A (Arg4Gln)	GG	945	118.5 ± 0.6		1125	126.6 ± 0.6		2070	122.6 ± 0.4	
		GA	519	118.8 ± 0.8		554	126.4 ± 0.8		1073	122.7 ± 0.6	
		AA	63	119.6 ± 2.3		85	125.9 ± 2.0		148	123.1 ± 1.6	
					.595			.752			.722
	344G>A	GG	1282	118.4 ± 0.5		1470	126.3 ± 0.5		2752	122.4 ± 0.4	
		GA	236	119.6 ± 1.2		281	126.9 ± 1.1		517	123.3 ± 0.8	
		AA	9	123.5 ± 6.2		14	132.8 ± 5.0		23	129.2 ± 3.9	
					.264			.342			.132
	20943T>A	TT	1351	118.8 ± 0.5		1558	126.5 ± 0.5		2909	122.7 ± 0.4	
		TA	169	117.0 ± 1.4		203	126.6 ± 1.3		372	121.8 ± 1.0	
		AA	6	121.6 ± 7.5		4	119.8 ± 9.4		10	120.3 ± 6.0	
					.336			.898			.328

*Protein C activities are expressed as a percentage of the activity in control plasma and presented as the mean ± SEM. *P* values were determined by analysis of covariance and adjusting for age, body mass index, smoking, alcohol consumption, diabetes mellitus, hypertension, and hyperlipidemia. SNP indicates single-nucleotide polymorphism.

respectively. Thus, our results suggest that *GGCX* 8016G>A (Arg325Gln) is associated with an increased plasma level of protein C in women, comparable to that seen in women with the *PROC* –1644G>A polymorphism.

4. Discussion

We performed systematic screening of genetic variation for 3 genes involved in the γ-carboxylation reaction required for blood homeostasis and examined the relationship between plasma protein C and protein S activities and SNP genotypes in a large Japanese population. The SNP *GGCX* 8016G>A, which leads to the substitution of Gln for Arg at amino acid residue 325, was significantly associated with variation in plasma protein C activity in women. Furthermore, the *VKORC1* 3730G>A polymorphism was significantly associated with differences in plasma protein S activity in the entire study population, and the *CALU* 20943T>A polymorphism was significantly associated with variation in plasma protein S activity in women.

Individuals with the *GGCX* 8016AA genotype had a 5.4% higher protein C activity than those with the GG genotype. A previously identified SNP in the *PROC* promoter, –1644G>A, was also associated with variation in protein C activity in our study population, and the difference in protein C activity between individuals with the GG and AA genotypes was 4.3%. Multivariate regression analyses indicated that both genetic polymorphisms were independently associated with protein C activity. Thus, the effect of *GGCX* 8016G>A on protein C activity was virtually the same, if not more pronounced,

Table 4.
Protein S Activity by *GGCX*, *VKORC1*, and *CALU* Genotype*

			Men			Women			Total		
Gene	SNP	Genotype	n	Protein S Activity, %	*P*	n	Protein S Activity, %	*P*	n	Protein S Activity, %	*P*
VKORC1	523G>A	GG	825	93.0 ± 0.7		948	83.2 ± 0.6		1773	88.0 ± 0.4	
		GA	40	88.7 ± 3.1		50	81.2 ± 2.4		90	84.9 ± 2.0	
		AA	0	—		0	—		0	—	
					.172			.407			.122
	1338A>G (His68Arg)	AA	857	92.8 ± 0.7		988	83.2 ± 0.6		1845	87.9 ± 0.4	
		AG	7	94.8 ± 7.4		8	82.5 ± 6.1		15	88.9 ± 4.8	
		GG	0	—		0	—		0	—	
					.786			.918			.832
	3730G>A	GG	740	93.2 ± 0.7		827	83.6 ± 0.6		1567	88.3 ± 0.5	
		GA	119	89.5 ± 1.8		164	81.3 ± 1.3		283	85.5 ± 1.1	
		AA	6	102.2 ± 7.9		7	69.2 ± 6.5		13	85.0 ± 5.2	
					.196			.023			.016
GGCX	412G>A	GG	634	92.6 ± 0.8		730	82.8 ± 0.6		1364	87.6 ± 0.5	
		GA	216	93.0 ± 1.3		245	84.1 ± 1.1		461	88.5 ± 0.9	
		AA	15	94.4 ± 5.0		23	85.1 ± 3.6		38	90.4 ± 3.0	
					.717			.246			.234
	8016G>A (Arg325Gln)	GG	436	92.4 ± 0.9		500	82.4 ± 0.8		936	87.4 ± 0.6	
		GA	357	93.2 ± 1.0		418	83.7 ± 0.8		775	88.3 ± 0.7	
		AA	72	93.1 ± 2.3		80	84.9 ± 1.9		152	88.5 ± 1.5	
					.581			.142			.282
	8445C>T	CC	774	93.2 ± 0.7		886	83.2 ± 0.6		1660	88.1 ± 0.5	
		CT	87	88.7 ± 2.1		107	82.4 ± 1.7		194	85.9 ± 1.3	
		TT	4	96.4 ± 9.7		5	85.4 ± 7.7		9	92.5 ± 6.2	
					.085			.740			.270
CALU	11G>A (Arg4Gln)	GG	545	93.0 ± 0.8		647	82.9 ± 0.7		1192	87.8 ± 0.5	
		GA	288	92.2 ± 1.2		300	83.2 ± 1.0		588	87.6 ± 0.8	
		AA	32	93.5 ± 3.4		51	86.0 ± 2.4		83	90.7 ± 2.0	
					.712			.350			.490
	344G>A	GG	733	92.7 ± 0.7		829	83.4 ± 0.6		1562	88.0 ± 0.5	
		GA	127	93.1 ± 1.7		159	81.5 ± 1.4		286	87.1 ± 1.1	
		AA	5	96.1 ± 8.7		10	88.6 ± 5.5		15	93.1 ± 4.8	
					.741			.507			.844
	20943T>A	TT	761	92.5 ± 0.7		873	83.7 ± 0.6		1634	88.1 ± 0.5	
		TA	100	95.3 ± 1.9		123	79.0 ± 1.6		223	86.6 ± 1.2	
		AA	3	78.7 ± 11.2		2	82.7 ± 12.1		5	76.4 ± 8.3	
					.407			.006			.130

*Protein S activities are expressed as a percentage of the activity in control plasma and presented as the mean ± SEM. *P* values were determined by analysis of covariance and adjusting for age, body mass index, smoking, alcohol consumption, diabetes mellitus, hypertension, and hyperlipidemia. SNP indicates single-nucleotide polymorphism.

than that seen for the *PROC* promoter polymorphism. Thus, these 2 genetic polymorphisms cooperatively influence interindividual variability in plasma protein C activity under normal conditions in subjects without known coagulopathies.

Although multiple studies have addressed the biological activities of vitamin K–dependent factors, the studies that have examined genetic factors influencing plasma levels of vitamin K–dependent proteins are limited. The Spanish family-based GAIT project identified a strong genetic component to variations in plasma protein C and protein S activities, and genetic polymorphisms were able to explain 22% to 50% of the observed differences in enzymatic activity [37]. A genome-wide linkage study explored quantitative trait loci influencing protein C and protein S levels in the serum and identified a region on chromosome 1q32 linked to free protein S levels. This study also identified the *NQO1* gene on chromosome 16 (16q23) as being associated with protein C levels [29,38]. We were not able to confirm this finding, likely because of differences in the study populations; the Spanish study was pedigree based and our study was population based. An additional analysis of quantitative trait loci for a protein C–deficient pedigree identified 3 loci, on chromosomes 11q23, 18p11.2-q11.2, and 10p12, as candidate regions for genes that increase the risk of venous thrombosis [39]. The *GGCX* gene lies on chromosome 2p12; thus, the significant association of the *GGCX* genotypes with plasma protein C activity described in the present report is the first data to identify chromosome 2p12 as an important locus affecting blood coagulation.

γ-Carboxylation occurs within the ER lumen. Residues Cys99 and Cys450 of GGCX are critical for its γ-carboxylase activity, and the region surrounding Leu394 is important for

Table 5.
Protein C Activity by *PROC* and *NQO1* Genotype*

Gene, SNP	Genotype	Men: Protein C Activity, %	Men: n	Women: Protein C Activity, %	Women: n	Total: Protein C Activity, %	Total: n
PROC,	CC	118.4 ± 1.5	154	124.5 ± 1.4	181	121.5 ± 1.0	335
−1657C>T	CT	118.5 ± 0.7	690	126.4 ± 0.7	734	122.5 ± 0.5	1424
(rs1799808)	TT	118.8 ± 0.7	679	126.9 ± 0.6	844	123.0 ± 0.5	1523
		P = .753		*P* = .150		*P* = .185	
PROC,	GG	116.2 ± 4.8	16	122.4 ± 4.0	22	119.1 ± 3.1	38
−1644G>A	GA	116.6 ± 1.1	321	123.5 ± 1.0	340	120.0 ± 0.7	661
(rs1799809)	AA	119.3 ± 0.6	1183	127.3 ± 0.5	1394	123.4 ± 0.4	2577
		P = .025		*P* = .001		*P* < .001	
NQO1,	CC	118.8 ± 1.3	202	125.5 ± 1.1	268	122.1 ± 0.9	470
2515C>T	CT	118.7 ± 0.7	751	126.9 ± 0.7	833	122.9 ± 0.5	1584
(rs1437135)	TT	118.6 ± 0.8	572	126.3 ± 0.7	665	122.5 ± 0.5	1237
		P = .890		*P* = .745		*P* = .922	

*Protein C activities are expressed as a percentage of the activity in control plasma and presented as the mean ± SEM. *P* values were obtained by multiple logistic analyses and adjusting for age, body mass index, percentages of smokers and drinkers, and percentages of individuals with diabetes mellitus, hypertension, and hyperlipidemia. SNP indicates single-nucleotide polymorphism.

enzyme/substrate interactions. The membrane topology of GGCX has been investigated with a tagging approach [5,6], and these residues are thought to reside in the ER lumen [40-42]. In contrast, another report has suggested that amino acid residues 343-355 mediate GGCX enzyme/substrate interactions and that the Cys-Val-Tyr sequence of residues 343 through 345 is necessary for both substrate binding and γ-carboxylase activity [43]. These protein regions are predicted to lie within the cytoplasmic region. The polymorphism responsible for the Arg325Gln mutation that we investigated in this study is likely located within the same cytoplasmic loop as residues 343 to 345, further suggesting the functional significance of this domain. Alternatively, other SNPs in linkage disequilibrium with the Arg325Gln mutation may influence γ-carboxylation; we observed 5 SNPs in tight linkage disequilibrium with this mutation (Table 1). Further studies are needed to fully characterize the *GGCX* encoded mutant protein with Gln325.

We identified large sex differences in the degree to which genotypic variation affects protein C and protein S activities. It is possible that hormonal and environmental factors substantially affect protein C and protein S activities in healthy individuals. Indeed, studies support this possibility [44,45]. It is well known that plasma protein S activity shows a strong sex difference. We previously reported that protein S activity was lower in women than in men by 22% and 23% in individuals aged 30 to 39 years and in those aged 40 to 49 years, respectively [46]. In addition, protein S activity in individuals aged 50 to 59 years was 16% lower in women than in men. If women with genotypes associated with lower protein S activity are in these age brackets, they may be at a greater risk of thrombosis. Thus, the genetic polymorphisms identified in the present study may have implications in clinical practice under these circumstances. Additionally, there were significant differences in our study in the proportions of men and women who consumed alcohol and smoked (Table 2). These lifestyle factors may also contribute to the differences in enzymatic activities we observed.

We have observed an association between SNPs in genes involved in γ-carboxylation and the activities of protein C and protein S in a Japanese population. These results provide valuable insight into the relationship between genetic variation in the γ-carboxylation system and interindividual variation in the levels and activities of vitamin K–dependent plasma proteins.

Table 6.
Multiple Regression Analyses for Protein C Activity in Women*

Parameter	Coefficient	*P*
Model with *GGCX* 8016G>A genotype (R^2 = 0.151)		
Age	2.56	<.001
Body mass index	3.19	<.001
Smoking	2.51	.200
Drinking	2.63	.013
Diabetes mellitus	3.03	.124
Hypertension	1.23	.234
Hyperlipidemia	10.21	<.001
GGCX 8016G>A genotype	4.46	.006
Model with *PROC* −1644G>A genotype (R^2 = 0.152)		
Age	2.48	<.001
Body mass index	3.19	<.001
Smoking	2.57	.189
Drinking	2.76	.009
Diabetes mellitus	2.72	.168
Hypertension	1.27	.219
Hyperlipidemia	10.21	<.001
PROC −1644G>A genotype	3.80	.001

*Diabetes mellitus was indicated by a fasting plasma glucose concentration ≥126 mg/dL or antidiabetic medication, hypertension was indicated by a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg or antihypertensive medication, and hyperlipidemia was indicated by total cholesterol level ≥220 mg/dL or antihyperlipidemia medication. *GGCX* 8016G>A genotypes were categorized as GG + GA or AA. *PROC* −1644G>A genotypes were categorized as GG + GA or AA.

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References

1. Vermeer C. γ-Carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. *Biochem J.* 1990;266:625-636.
2. Furie B, Bouchard BA, Furie BC. Vitamin K-dependent biosynthesis of γ-carboxyglutamic acid. *Blood.* 1999;93:1798-1808.
3. Furie B, Furie BC. Molecular basis of vitamin K-dependent γ-carboxylation. *Blood.* 1990;75:1753-1762.
4. Stafford DW. The vitamin K cycle. *J Thromb Haemost.* 2005;3:1873-1878.
5. Wu SM, Cheung WF, Frazier D, Stafford DW. Cloning and expression of the cDNA for human γ-glutamyl carboxylase. *Science.* 1991;254:1634-1636.
6. Tie J, Wu SM, Jin D, Nicchitta CV, Stafford DW. A topological study of the human γ-glutamyl carboxylase. *Blood.* 2000;96:973-978.
7. Rost S, Fregin A, Ivaskevicius V, et al. Mutations in *VKORC1* cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature.* 2004;427:537-541.
8. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature.* 2004;427:541-544.
9. Tie JK, Nicchitta C, von Heijne G, Stafford DW. Membrane topology mapping of vitamin K epoxide reductase by in vitro translation/cotranslocation. *J Biol Chem.* 2005;280:16410-16416.
10. Wajih N, Sane DC, Hutson SM, Wallin R. Engineering of a recombinant vitamin K-dependent γ-carboxylation system with enhanced γ-carboxyglutamic acid forming capacity: evidence for a functional *CXXC* redox center in the system. *J Biol Chem.* 2005;280:10540-10547.
11. Geisen C, Watzka M, Sittinger K, et al. VKORC1 haplotypes and their impact on the inter-individual and inter-ethnical variability of oral anticoagulation. *Thromb Haemost.* 2005;94:773-779.
12. Wallin R, Sane DC, Hutson SM. Vitamin K 2,3-epoxide reductase and the vitamin K-dependent γ-carboxylation system. *Thromb Res.* 2002;108:221-226.
13. D'Andrea G, D'Ambrosio RL, Di Perna P, et al. A polymorphism in the *VKORC1* gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. Blood. 2005;105:645-649.
14. Rieder MJ, Reiner AP, Gage BF, et al. Effect of *VKORC1* haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med.* 2005;352:2285-2293.
15. Bodin L, Verstuyft C, Tregouet DA, et al. Cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*) genotypes as determinants of acenocoumarol sensitivity. *Blood.* 2005;106:135-140.
16. Yuan HY, Chen JJ, Lee MT, et al. A novel functional VKORC1 promoter polymorphism is associated with inter-individual and inter-ethnic differences in warfarin sensitivity. *Hum Mol Genet.* 2005;14:1745-1751.
17. Wadelius M, Chen LY, Downes K, et al. Common *VKORC1* and *GGCX* polymorphisms associated with warfarin dose. *Pharmacogenomics J.* 2005;5:262-270.
18. Sconce EA, Khan TI, Wynne HA, et al. The impact of *CYP2C9* and *VKORC1* genetic polymorphism and patient characteristics upon warfarin dose requirements: proposal for a new dosing regimen. *Blood.* 2005;106:2329-2333.
19. Mushiroda T, Ohnishi Y, Saito S, et al. Association of *VKORC1* and *CYP2C9* polymorphisms with warfarin dose requirements in Japanese patients. *J Hum Genet.* 2006;51:249-253.
20. Marsh S, King CR, Porche-Sorbet RM, Scott-Horton TJ, Eby CS. Population variation in *VKORC1* haplotype structure. *J Thromb Haemost.* 2006;4:473-474.
21. Takahashi H, Wilkinson GR, Nutescu EA, et al. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. *Pharmacogenet Genomics.* 2006;16:101-110.
22. Yabe D, Nakamura T, Kanazawa N, Tashiro K, Honjo T. Calumenin, a Ca^{2+}-binding protein retained in the endoplasmic reticulum with a novel carboxyl-terminal sequence, HDEF. *J Biol Chem.* 1997;272:18232-18239.
23. Wallin R, Hutson SM, Cain D, Sweatt A, Sane DC. A molecular mechanism for genetic warfarin resistance in the rat. *FASEB J.* 2001;15:2542-2544.
24. Wajih N, Sane DC, Hutson SM, Wallin R. The inhibitory effect of calumenin on the vitamin K-dependent γ-carboxylation system: characterization of the system in normal and warfarin-resistant rats. *J Biol Chem.* 2004;279:25276-25283.
25. Esmon CT. Inflammation and thrombosis. *J Thromb Haemost.* 2003;1:1343-1348.
26. Dahlback B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. *Arterioscler Thromb Vasc Biol.* 2005;25:1311-1320.
27. Spek CA, Koster T, Rosendaal FR, Bertina RM, Reitsma PH. Genotypic variation in the promoter region of the protein C gene is associated with plasma protein C levels and thrombotic risk. *Arterioscler Thromb Vasc Biol.* 1995;15:214-218.
28. Aiach M, Nicaud V, Alhenc-Gelas M, et al. Complex association of protein C gene promoter polymorphism with circulating protein C levels and thrombotic risk. *Arterioscler Thromb Vasc Biol.* 1999;19:1573-1576.
29. Buil A, Soria JM, Souto JC, et al. Protein C levels are regulated by a quantitative trait locus on chromosome 16: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project. *Arterioscler Thromb Vasc Biol.* 2004;24:1321-1325.
30. Mannami T, Baba S, Ogata J. Potential of carotid enlargement as a useful indicator affected by high blood pressure in a large general population of a Japanese city: the Suita Study. *Stroke.* 2000;31:2958-2965.
31. Kokubo Y, Kamide K, Inamoto N, et al. Identification of 108 SNPs in *TSC, WNK1,* and *WNK4* and their association with hypertension in a Japanese general population. *J Hum Genet.* 2004;49:507-515.
32. Sakata T, Okamoto A, Mannami T, Matsuo H, Miyata T. Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese. *J Thromb Haemost.* 2004;2:528-530.
33. Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. *J Thromb Haemost.* 2004;2:1012-1013.
34. Okuda T, Fujioka Y, Kamide K, et al. Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet.* 2002;47:387-394.
35. Antonarakis SE, and the Nomenclature Working Group. Recommendations for a nomenclature system for human gene mutations. *Hum Mutat.* 1998;11:1-3.
36. Tanaka C, Kamide K, Takiuchi S, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res.* 2003;26:301-306.

37. Souto JC, Almasy L, Blangero J, et al. Genetic regulation of plasma levels of vitamin K-dependent proteins involved in hematostatis: results from the GAIT Project. Genetic Analysis of Idiopathic Thrombophilia. *Thromb Haemost.* 2001;85:88-92.
38. Almasy L, Soria JM, Souto JC, et al. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project. *Arterioscler Thromb Vasc Biol.* 2003;23:508-511.
39. Hasstedt SJ, Scott BT, Callas PW, et al. Genome scan of venous thrombosis in a pedigree with protein C deficiency. *J Thromb Haemost.* 2004;2:868-873.
40. Pudota BN, Miyagi M, Hallgren KW, et al. Identification of the vitamin K-dependent carboxylase active site: Cys-99 and Cys-450 are required for both epoxidation and carboxylation. *Proc Natl Acad Sci U S A.* 2000;97:13033-13038.
41. Mutucumarana VP, Acher F, Straight DL, Jin DY, Stafford DW. A conserved region of human vitamin K-dependent carboxylase between residues 393 and 404 is important for its interaction with the glutamate substrate. *J Biol Chem.* 2003;278:46488-46493.
42. Tie JK, Mutucumarana VP, Straight DL, Carrick KL, Pope RM, Stafford DW. Determination of disulfide bond assignment of human vitamin K-dependent γ-glutamyl carboxylase by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Biol Chem.* 2003;278:45468-45475.
43. Pudota BN, Hommema EL, Hallgren KW, McNally BA, Lee S, Berkner KL. Identification of sequences within the γ-carboxylase that represent a novel contact site with vitamin K-dependent proteins and that are required for activity. *J Biol Chem.* 2001;276:46878-46886.
44. Tait RC, Walker ID, Islam SI, et al. Protein C activity in healthy volunteers: influence of age, sex, smoking and oral contraceptives. *Thromb Haemost.* 1993;70:281-285.
45. Henkens CM, Bom VJ, Van der Schaaf W, et al. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. *Thromb Haemost.* 1995;74:1271-1275.
46. Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis among Japanese: importance of protein S K196E mutation. *Int J Hematol.* 2006;83:217-223.

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REGULAR ARTICLE

Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population

Shoko Sugiyama [a,b], Hisao Hirota [a,✠], Rina Kimura [b], Yoshihiro Kokubo [c], Tomio Kawasaki [d,*], Etsuji Suehisa [e], Akira Okayama [c], Hitonobu Tomoike [c], Tokio Hayashi [f], Kazuhiro Nishigami [f], Ichiro Kawase [g], Toshiyuki Miyata [b]

[a] *Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita City, Osaka 565-0871, Japan*
[b] *Research Institute, National Cardiovascular Center, Japan*
[c] *Department of Preventive Cardiology, National Cardiovascular Center, Japan*
[d] *Cardiovascular Surgery, Osaka University Graduate School of Medicine, Japan*
[e] *Department of Laboratory Medicine, Osaka University Hospital, Japan*
[f] *Department of Cardiology, National Cardiovascular Center, Japan*
[g] *Respiratory Medicine and Rheumatic Diseases, Osaka University Graduate School of Medicine, Japan*

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Abstract

Introduction: Thrombomodulin (TM) is an essential cofactor in protein C activation by thrombin. Here, we evaluated the contribution of genetic variations in the TM gene to soluble TM (sTM) level and deep vein thrombosis (DVT) in Japanese.
Patients and methods: We sequenced the TM putative promoter, exon, and 3′-untranslated region in DVT patients (n=118). Among 17 genetic variations we identified, two missense mutations (R385K, D468Y) and three common single nucleotide polymorphisms (−202G>A, 2487A>T, 2729A>C) were genotyped in a general population of 2247 subjects (1032 men and 1215 women) whose sTM levels were measured. We then compared the frequency of these mutations in DVT patients

Abbreviations: DVT, deep vein thrombosis; TM, thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S; EGF, epidermal growth factor; SNP, single-nucleotide polymorphism; sTM, soluble TM; 5′-UTR, 5′-untranslated region; 3′-UTR, 3′-untranslated region.

* Corresponding author. Tel.: +81 6 6879 3251; fax: +81 6 6879 3259.
E-mail address: kawasaki@sug2.med.osaka-u.ac.jp (T. Kawasaki).
✠ Deceased.

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