

**Table 3.**

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

	No. of heterozygotes (prevalence)		Odds ratio, 95% CI (vs general population)	P
	Patients with DVT (n = 108)	General population (n = 4517)		
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
$\chi^2$	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  $\alpha_1$ -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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Original Article

## Novel compound heterozygote mutations (H234Q/R1206X) of the *ADAMTS13* gene in an adult patient with Upshaw–Schulman syndrome showing predominant episodes of repeated acute renal failure

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### Abstract

**Background.** Unlike acquired thrombotic thrombocytopenic purpura or haemolytic uraemic syndrome, which are often intractable, thrombotic microangiopathy in patients with Upshaw–Schulman syndrome (USS) – a congenital deficiency of von Willebrand factor-cleaving protease (ADAMTS13) activity – responds very well to plasma infusion and does not even require plasma exchange. However, the symptoms significantly vary in each individual and thus clinicians often overlook this diagnosis.

**Methods.** A 31-year-old adult male patient with thrombotic microangiopathy, which was complicated with repeated episodes of acute renal failure, is reported. We suspected that the patient had USS and performed assays of ADAMTS13 activity and its inhibitor, followed by *ADAMTS13* gene analysis of the patient and his parents.

**Results.** The patient had extremely low ADAMTS13 activity and has no inhibitors of ADAMTS13. Through an *ADAMTS13* gene analysis of this family, we found two novel mutations responsible for the disease: a missense mutation in exon 7 [702 C → A (H234Q)] from the father and a nonsense mutation in exon 26 [3616 C → T (R1206X)] from the mother.

**Conclusions.** Our experience appears to indicate the importance of assays of ADAMTS13 activity and its inhibitor in patients who have episodes of renal insufficiency in association with thrombotic microangiopathy, for diagnosis and choice of treatment.

**Keywords:** acute renal failure; genetic disorder; haemolytic uraemic syndrome; thrombotic microangiopathy; von Willebrand factor

### Introduction

Thrombotic thrombocytopenic purpura (TTP) and haemolytic uraemic syndrome (HUS) are both categorized within thrombotic microangiopathy (TMA), featured by microangiopathic haemolytic anaemia with thrombocytopenia [1]. The term TTP typically refers to a form of TMA that affects adolescents and adults and predominantly causes central nervous system disorders, whereas HUS refers to TMA, which mainly involves kidney and typically affects young children with diarrhoea caused by *Escherichia coli* O157:H7 infection. TTP and HUS are mostly indistinguishable by laboratory findings and pathology and thus in clinical practice are often referred to as HUS/TTP [2]. It has, however, been recognized that some forms of TTP respond well to plasma infusion (PI) [3] or plasma exchange (PE), whereas typical diarrhoea-associated HUS does not [4]. Recently, the plasma activity of von Willebrand factor (VWF)-cleaving protease (ADAMTS13, a disintegrin and metalloprotease domain, with thrombospondin type 1 motif 13) was found to be deficient in an inherited form of TTP, which differs from acquired idiopathic TTP characterized by its neutralizing or non-neutralizing inhibitor and from acquired HUS by subnormal enzyme activity [4–6]. If the onset of this inherited form of TTP is in the neonatal period, it is alternatively called Upshaw–Schulman

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syndrome (USS), although there is also an adult onset form of the disease [7].

## Subjects and methods

### Patient

A 31-year-old male was admitted to the nephrology service for acute renal failure, microangiopathic haemolytic anaemia and thrombocytopenia.

He had a history of moderate jaundice as a newborn, which was treated with phototherapy. At 3 months of age, he had episodes of purpura and thrombocytopenia after diphtheria/pertussis/tetanus immunization, for which he was diagnosed with idiopathic thrombocytopenic purpura. Since the age of 2 years, he had an episode of intracranial bleeding after minor head injury and several episodes of high-renin hypertension and acute renal failure complicated with TMA, all of which were successfully treated with PI. Since then he has been treated with PI every other week to maintain blood platelet count  $>2 \times 10^9/l$ , by which he was diagnosed by a previous paediatrician with recurrent HUS/TTP of unknown aetiology.

On admission, he showed no neurological deficits or any other specific symptoms, but subsequently his serum creatinine progressively rose from 0.87 to 5.78 mg/dl with increased LDH (1968 IU/l), low platelet count ( $3.5 \times 10^9/l$ ) and the presence of schistocytes. However, with PI, LDH rapidly fell back to normal level and his renal dysfunction and thrombocytopenia gradually resolved. He had no apparent family history of congenital coagulopathy, but his elder brother, who died at the age of 2 years, had laboratory data compatible with TMA, which raised our suspicion of the presence of hereditary HUS/TTP, such as USS.

We subsequently performed assays of ADAMTS13 activity, followed by *ADAMTS13* gene analysis of the patient and his parents.

### Assay of ADAMTS13 activity and its inhibitor

Plasma ADAMTS13 activity was assayed by the method of Furlan *et al.* [5] based on VWF multimer analysis, with a slight modification as described before [8]. The ADAMTS13 activity of pooled normal plasma was defined as 100%. The normal range of ADAMTS13 activity was  $102 \pm 23\%$  (mean  $\pm$  SD) [9].

The inhibitor activity against ADAMTS13 was measured as described by Furlan *et al.* [10] based on the Bethesda method [3]. One unit of inhibitor was defined as the amount that reduced the ADAMTS13 activity to 50% of the control.

### ADAMTS13 gene analysis

After obtaining approval from the ethics committees of both the sample-collecting hospital and gene analysis institute, and informed consent, *ADAMTS13* genes of the patient and his parents were analysed by DNA sequencing. All 29 exons and exon-intron boundary sites of the *ADAMTS13* gene were amplified and sequenced as recently described [11].

## Results

ADAMTS13 activity of the patient's plasma, which was obtained on admission (and just before PI), was  $<3\%$  of the pooled normal plasma (Figures 1 and 2) and its inhibitor was undetectable ( $<0.5$  Bethesda U/ml). Three novel mutations were identified (Figure 1). The patient had compound heterozygous mutations comprised of a missense mutation in exon 7 [702 C  $\rightarrow$  A (H234Q)] inherited from his father and a nonsense mutation in exon 26 [3616 C  $\rightarrow$  T (R1206X)] inherited from his mother. His father had an additional missense mutation at exon 21 [2708 C  $\rightarrow$  T (S903L)], but its effect on ADAMTS13 activity is presently unknown [11,12]. Thus, on this occasion, a solid diagnosis of USS was made. Although the patient's parents had heterozygous mutations and relatively low ADAMTS13 activity (father: 24%, mother: 21%; Figure 2), they had no episode of haematological problems and, to date, have been in good health. To investigate the frequencies of these mutations, we sequenced the relevant exons of ADAMTS13 in

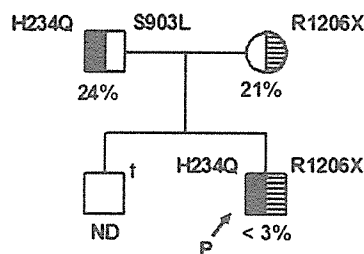
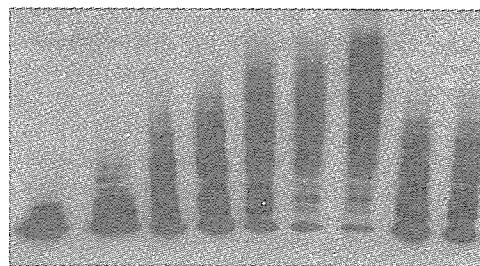


Fig. 1. Pedigree of the index patient with gene haplotypes and plasma activity of ADAMTS13. The circle represents a female and squares represent males. Plasma ADAMTS13 activity (%) is shown under the circle and the squares. Mutations found in the *ADAMTS13* gene are shown as one-letter amino acid abbreviations numbered from the initial Met codon. The arrow indicates the index patient. Both the mother and the father of the index patient are asymptomatic carriers. † A deceased individual. P, index patient; ND: value not determined.



ADAMTS13 Activity (%) 100 50 25 12.5 6.3 3.1 <3 21 24  
Case Mother Father

Fig. 2. Cleavage of VWF multimer by ADAMTS13. Quantitative assay of plasma ADAMTS13 activity from a control, the patient and the parents. Following incubation of VWF, plasma from six dilution series of a normal control and plasma from the patient and his parents, sodium dodecyl sulphate-agarose gel electrophoresis and western blotting were performed as shown in a previous report [8]. The plasma ADAMTS13 activity was shown as a percentage of the normal control.

the general Japanese population. Seven heterozygotes carrying S903L were found in 64 subjects, suggesting that the mutation is a common polymorphism with an allele frequency of 5.5%. On the other hand, both H234Q and R1206X mutations were excluded as common polymorphisms by the screening of 64 subjects.

## Discussion

USS, which may be alternatively called congenital chronic relapsing TTP or familial HUS/TTP, is characterized by repeated episodes of thrombocytopenia and haemolytic anaemia which ameliorate quickly by PI [13]. Typically, patients have episodes of hyperbilirubinaemia with negative Coombs test during infancy followed by repeated episodes of TTP [14]. In 1997, Furlan *et al.* [10] reported four cases of chronic relapsing TTP lacking ADAMTS13 activity. Since then, ADAMTS13 has been known to have a central pathogenic role in TTP and a defect in its gene was reported to cause USS, which is a form of TTP with a neonatal onset, although there is also an adult onset disease caused by *ADAMTS13* gene defect [15,7]. Patients with USS either have homozygous or compound heterozygous gene mutations [11,12,15]. Another familial or inherited form of HUS/TTP is associated with factor H deficiency. Patients with factor H deficiency have, predominantly, renal impairment and low level of serum C3. Factor H deficiency seems to aggravate C3 activation, which potentiates autoantibody-mediated or immune complex-mediated glomerular injury. While some patients with factor H deficiency were reported to accompany low ADAMTS13 level and renal involvement [16], our case showed normal factor H level (data not shown).

In the present study, we identified two novel mutations, H234Q in exon 7 and R1206X in exon 26, in the *ADAMTS13* gene. The missense mutation, H234Q, resides in the metalloprotease domain. The conserved active site sequence HEXXHXXGXXHD (amino acids 224–235) in this domain contains 3 His residue that coordinate the catalytic Zn<sup>2+</sup> ion [17]. It is a reasonable assumption that if one His residue is replaced with Gln, the mutant lacks its protease activity. The nonsense mutation, R1206X, resides in the CUB1 domain of the *ADAMTS13* gene. This mutant lacks 222 amino acids in the C-terminal that include a part of the CUB1 domain and the entire CUB2 domain. The deletion of these regions may cause secretion defects during synthesis. It has been suggested that the CUB domains are important for interactions between ADAMTS13 and VWF [18]. Therefore, deletion of the C-terminal region may affect substrate recognition. In the previous study, we sequenced exon 7 in 364 Japanese individuals and found no H234Q mutation [9]. The R1206X mutation was not observed in

the 12 Japanese families with USS so far determined in our laboratory [11,12]. However, recombinant ADAMTS13 reproducing the patient's mutations has not been performed; the above-mentioned mechanisms of inducing ADAMTS13 deficiency are only speculative at this point.

Unusually large VWF multimers (UL-VWFMs) are secreted from vascular endothelial cells. UL-VWFMs tend to aggregate platelets to form UL-VWFM–platelet complexes, which may embolize microvessels and cause organ ischaemia. ADAMTS13 cleaves UL-VWFMs to a series of smaller multimers, which lack the tendency to aggregate platelets. Therefore, loss of ADAMTS13 activity caused either by genetic defects (USS) or by its inhibitors causes intravascular platelet aggregation, leading to TMA.

Thus, theoretically, early intervention with PE or PI should be effective in these disorders by supplementing ADAMTS13 with or without removal of its inhibitors, respectively. In fact, there has been evidence that extremely low levels of ADAMTS13 activity were associated with excellent response to PE and good prognosis [12,17], although there also are reports challenging this view [2]. Our experience of 20 patients with USS indicates that all are doing quite well with regular infusion of plasma, except one patient who died of TTP that became refractory to plasma after cholecystectomy.

Patients with TMA tend to be labelled as having TTP if clinical features are predominated by neurological dysfunction or HUS if renal impairment is the predominant feature. However, renal impairment accompanies as much as 44% of patients with TTP [19]. Even if the predominant feature of these patients with TMA is renal dysfunction, we need to consider diagnosis of TTP and check ADAMTS13 activity, especially if an inherited form of the disease is suspected. If ADAMTS13 activity is very low, PE in patients with inhibitors or PI in cases with USS would be justified and should be done as early as possible. Tsai has proposed that the definition of TTP should be a pro-thrombotic state in the microvasculature caused by severe ADAMTS13 deficiency, such that we can easily identify a group of patients who have a common pathogenic mechanism for the future enzyme replacement or gene therapy [4]. Although some challenge this notion, it is still important to develop rapid and simple assay methods for ADAMTS13 activity so that the appropriate therapy can be given immediately.

Our experience appears to indicate that assays of ADAMTS13 activity and its inhibitor should be considered in patients who have repeated episodes of renal insufficiency as well as thrombocytopenia.

*Acknowledgements.* The case of the patient was presented briefly in a review paper published in *Hypertension Research* journal [20].

*Conflict of interest statement.* None declared.

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

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### Abstract

$\gamma$ -Glutamyl carboxylation, a reaction essential for the activity of vitamin K–dependent proteins, requires the concerted actions of  $\gamma$ -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 ( $130.8\% \pm 1.5\%$ ,  $n = 156$ ) was significantly greater ( $P = .002$ ) than that of individuals with either the AG ( $126.8\% \pm 0.7\%$ ,  $n = 728$ ) or the AA ( $125.4\% \pm 0.6\%$ ,  $n = 881$ ) 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  $\gamma$ -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;  $\gamma$ -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  $\gamma$ -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  $\gamma$ -carboxyl modification enables calcium binding, which is essential for the physiological action of these proteins [3].

$\gamma$ -Glutamyl carboxylase (GGCX) and the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR) mediate vitamin K–dependent  $\gamma$ -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  $\gamma$ -carboxylation reaction [10,12]. GGCX catalyzes the addition of carbon dioxide

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to Gla 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  $\text{Ca}^{2+}$ -binding proteins [22]. A recent study suggested that CALU inhibits the  $\gamma$ -carboxylation reaction [23]. Small interfering RNA-mediated CALU knock-down caused a 5-fold increase in  $\gamma$ -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].  $\text{Ca}^{2+}$  binding to the Gla domain of protein C and protein S is essential for their anticoagulant activities.

We hypothesized that polymorphisms in genes controlling  $\gamma$ -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 check-ups. Diabetes mellitus was defined as a fasting blood glucose level  $\geq 126$  mg/dL or current use of insulin or oral antidiabetic agents. Hypertension was diagnosed by a systolic blood

pressure  $\geq 140$  mm Hg, a diastolic blood pressure  $\geq 90$  mm Hg, or current use of antihypertensive medications. Hyperlipidemia was defined as a total cholesterol level  $\geq 220$  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 (Stacloct 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  $\chi^2$  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, GGX, 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	tctcctccaggtgtgacacgg[G/T] agtgggaggcgtggggcctc			
	403G>T	A		Intron 1	0.995	0.005	attattccgggactcgcac[G/T] tgaattggatgccaaggaat			
	523G>A			Intron 1	0.968	0.032	ggggtgttggcgtggctgc[G/A] cccaggaacaagggtggccc	Done		
	859A>T			Intron 1	0.995	0.005	ctaccaaaaatagaaaatt[A/T] atcgggcgctatggcgggtg			
	1173C>T	B		Intron 1	0.036	0.966	ggtgccaggagatcatcgcac[C/T] cttgactagtagggagggt		rs9934438	
	1338A>G		His68Arg	Exon 2	0.995	0.005	gggtttcgggctgtggagc[A/G] tgtgtcgggacaggacagca	Done		
	1411A>G	A	Leu92	Exon 2	0.995	0.005	atcttctacactacagct[A/G] ttgttaggtgagtggtctccg			
	1446insGCCCC			Intron 2	0.995	0.005	ctccgccccctcctgcc[----/ Gcccc]gccccccccctcatcc			
	1542G>C	B		Intron 2	0.046	0.954	gccagctagctgctcatcac[G/C] gagcgtcctcgggtgggga		rs8050894	
	2255T>C	B		Intron 2	0.957	0.043	ctgaggaggcccagcacca[T/C] ggtcctggctgacacatggt		rs2359612	
	3730G>A	B		Exon 3	0.958	0.042	ccctcctcctgccatacc[G/A] cacatgacaatggaccaa	Done	rs7294	
	3839G>A			Exon 3	0.990	0.010	ctcttccattgccaggga[G/A] ggaaggttctgagcaataaa			
	GGX	-310A>C			Promoter	0.995	0.005	aaggaagttgggaaactcag[A/C] ctctttcagtgtttaaaaa		
		-222G>T			Promoter	0.995	0.005	tggagatcaacggcactaag[G/T] ggtaccttggcccgtcca		
-187G>T				5' UTR	0.989	0.011	gtccaaggctcctgttcc[G/T] ccccctgcccaagcaagg			
-101G>A				5' UTR	0.989	0.011	gcttggggcggccgcaca[G/A] ccgctgacgcgtcgggaggc			
-70G>A		C		5' UTR	0.995	0.005	gtcgggaggCggagcctagg[G/A] aagcaaattcctctggcggc			
15C>T			Ala5	Exon 1	0.995	0.005	agagcaatggcgtgtctgc[C/T] gggtccgcggacctgcc			
150delT		D		Intron 1	0.646	0.354	ccggtgaggcggggggggg[T/-] cctctgtgggaagggggcg		rs11350741	
282G>T		D		Intron 1	0.634	0.366	gacattagggatcgtggag[G/T] ggggctgcaactgcaggatg		rs10172544	
307C>T				Intron 1	0.995	0.005	ctgcactgcaggatgtttc[C/T] tatagtcagatcattgaggc			
377A>T		D		Intron 1	0.634	0.366	aagctctgagctgttggctc[A/T] gtgatttcttgattgagg		rs7568458	
412G>A				Intron 1	0.860	0.140	ttgaggtggagcccacccc[G/A] cacacatatttctccattt	Done	rs1254898	
2478C>T		C	Asp113	Exon 3	0.995	0.005	ctacgccactgccactga[C/T] tggatgtatctgtctacac		rs6751560	
5165G>A				Intron 4	0.995	0.005	caatgtttacctccctcgg[G/A] taggtctgtgacggctctgc			
5424A>C		D		Intron 5	0.630	0.370	aatcccttaactctgaatt[A/C] tcttattgtcaaggaaaga		rs6738645	
5697A>G				Intron 5	0.989	0.011	ataagtagattattgggtc[A/G] aggactgtaatattgggtc			
6146A>G			Intron 6	0.995	0.005	gctctgggagatatatgg[G/A] tagataggcaagaaggaaa				
6972(CAA) <sub>8-12</sub>			Intron 6	—	—	gacagagtgaactctgtt[(CAA) <sub>8-12</sub> ] acaacctagaggagtgtctt		rs10654848		

Continued

Table 1  
Continued

8016G>A	D	Arg325Gln	Exon 8	0.693	0.307	gctggtgtcctactgcccc[G/A] aagggtgcaacaactgttg	Done	rs699664
8021T>C	D	Leu327	Exon 8	0.307	0.693	tgtcctactgccccgaagg[T/C] tgcaacaactgttgcccctc		
8445C>T		Thr414	Exon 9	0.938	0.063	caccagcagtggaagatcac[C/T] taccgtgatggccgcactgg	Done	rs10179904
8838G>A			Intron 9	0.995	0.005	agttgataatgatgagggtgg[G/A] actaatatgggggtttgggg		
9078C>T			Intron 10	0.995	0.005	ttttaccaagatgaacagc[C/T] ctgctcctgggtattgtct		
9342-9344delCTT	D		Intron 10	0.741	0.259	catgctgacacagccattt[Ctt/---] ctattccctgctcttaggaa		
9654C>T			Intron 11	0.995	0.005	ggagaggcttttctgtctt[C/T] cttctggcttcttgccag		
9980-9981insA	C		Intron 11	0.995	0.005	gggattaacaacagcagaaa[-/A] gaactgagtgcagtagctca		
10911G>A			Intron 14	0.989	0.011	atttgcattgccatcatat[G/A] ttggcaagcttgtaacttt		
11609-11610insG			3' UTR	0.984	0.016	ttttttttttttgggggg[-/G] cggggttctaaagctgtt		
12062-12063 insTAAA	D		3' UTR	0.630	0.370	tctcaaaataaataaataa[---/ taaa]gtggctcttgggaaaagcaa		rs5832649
12105C>G	C		3' UTR	0.995	0.005	taatgtaccacgatgaatag[C/G] taactgtccaagtgtttg		
12175G>C			3' UTR	0.989	0.011	tacctgattattacattag[G/C] ctgagaggtaaaataattg		
CALU -9755G>T	E		Promoter	0.978	0.022	ccagagcgcaagatgagggg[G/T] agtgtgggagatggagccta		
-9459(GGGGCT) <sub>3,7</sub>			Promoter	—	—	gccccacgcccccagctac[(GGGGCT) <sub>3,7</sub> ] ggagcccgccgtccaacaa		
-9429G>T	E		Promoter	0.979	0.021	gtccaacaatcccggggac[G/T] agagttggcggaatccgc		rs7795071
-9171T>C			5' UTR	0.974	0.026	gcggccacggcatcctgtgc[T/C] gtgggggctacgaggaaagg		
-97G>A	F		Intron 1	0.927	0.073	atatgtatatcaaatata[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	aattatttctccttgcag[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	ctgtatatgctataataagta[T/C] tggccagattcagaatccaa		
11484G>A			Intron 5	0.990	0.010	aattagttgggtgtgtggc[G/A] ggcgccctgtaatcccagcta		
18631A>G	H		Intron 5	0.989	0.011	atttctgatttattaagggt[A/G] taagaactctttgctctcat		
18642-18643delTT			Intron 5	0.995	0.005	tattaaggataagaactct[TT/--] gctctcatgtgacttaaaa		
18776A>T			Intron 5	0.995	0.005	gtagctgctgttgggtcca[A/T] acaaatggatgaggaaaaag		
20588A>G	F		3' UTR	0.932	0.068	ggcatgatgagttctgagct[A/G] cggaggaaacctcatttct		rs1043550
20674C>T	I		3' UTR	0.995	0.005	ttcgctactgagactgta[C/T] tacaacttttaagacatg		

Continued

Table 1  
Continued

20943T>A	F	3' UTR	0.927	0.073	ttactcttctcaaccctt[T/A] tatgattttaataattctca	Done	rs11653
21375G>A	F	3' UTR	0.927	0.073	aaactaaaggaaaaatacaa[G/A] Jtgtttcggggcatacattt		rs1043595
22706-22707insT	G	3' UTR	0.990	0.010	ccctttgaaattttttt[-/T] gtttgtttaaatacaagc		
22983C>T	F	3' Flanking	0.926	0.074	gtcagaatgggaactctct[C/T] gaagtctcccaactcaga		rs1044226
23087C>T	F	3' Flanking	0.937	0.063	ccttctcaccacagcataa[C/T] cctttttacttccattagg		
23194G>A		3' Flanking	0.005	0.995	atgtacacacagtttatct[G/A] tagagcataaggtaaaacaa		
23340A>T	I	3' Flanking	0.995	0.005	taagacaaataaaagttt[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  $\pm$  SEM) in women was 125.5%  $\pm$  0.7% ( $n = 881$ ) with the GG genotype, 126.7%  $\pm$  0.8% ( $n = 728$ ) with the GA genotype, and 130.6%  $\pm$  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  $\pm$  SEM) was 83.6%  $\pm$  0.6% ( $n = 827$ )

in women with the *VKORC1* 3730GG genotype, 81.6%  $\pm$  1.4% (n = 164) for those with the GA genotype, and 68.3%  $\pm$  6.6% (n = 7) for women with the AA genotype, whereas these values were 83.7%  $\pm$  0.6% (n = 873) in women with the *CALU* 20943TT genotype, 79.2%  $\pm$  1.6% (n = 123) for the TA genotype, and 82.5%  $\pm$  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%  $\pm$  1.5% [mean  $\pm$  SEM], n = 156) than in women with the GA genotype (126.8%  $\pm$  0.7%, n = 728) or the GG genotype (125.4%  $\pm$  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%  $\pm$  1.9% [mean  $\pm$  SEM], n = 80) in *GGCX* 8016G>A (Arg325Gln) tended to exhibit higher protein S activities than those with either the GA genotype (83.7%  $\pm$  0.8%, n = 418) or the GG genotype (82.4%  $\pm$  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%  $\pm$  0.6%, n = 827) than women with the GA genotype (81.3%  $\pm$  1.1%, n = 164) or the AA genotype (69.2%  $\pm$  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%  $\pm$  0.6%, n = 873) than women with either the TA genotype (79.0%  $\pm$  1.6%, n = 123) or the AA genotype (82.7%  $\pm$  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 occur-

**Table 2.**

Subject Characteristics\*

	Men (n = 1709)	Women (n = 1946)	P
Age, y	66.1 $\pm$ 11.3	63.5 $\pm$ 11.1	<.0001
Body mass index, kg/m <sup>2</sup>	23.3 $\pm$ 3.0	22.4 $\pm$ 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 $\pm$ 0.10	0.90 $\pm$ 0.07	.0232
Protein C activity, %†	119 $\pm$ 20	126 $\pm$ 21	<.0001
Protein S activity, %‡	92.8 $\pm$ 21.2	83.2 $\pm$ 17.6	<.0001

\*Age, body mass index, prothrombin time, and protein C and protein S activities are presented as the mean  $\pm$  SD. Diabetes mellitus was indicated by a fasting plasma glucose level  $\geq$ 126 mg/dL or antidiabetic medication, hypertension was indicated by a systolic blood pressure  $\geq$ 140 mm Hg and/or a diastolic blood pressure  $\geq$ 90 mm Hg or antihypertensive medication, and hyperlipidemia was indicated by a total cholesterol level  $\geq$ 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.

rence 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.

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  $\pm$  SEM) was 119.1%  $\pm$  3.1% (n = 38), whereas it was 120.0%  $\pm$  0.7% (n = 661) for individuals with the GA genotype and 123.4%  $\pm$  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\*

Gene	SNP	Genotype	Men			Women			Total					
			n	Protein C Activity, %	P	n	Protein C Activity, %	P	n	Protein C Activity, %	P			
<i>VKORC1</i>	523G>A	GG	1454	118.7 ± 0.5	.454	1672	126.4 ± 0.5	.526	3126	122.6 ± 0.3	.914			
		GA	72	117.1 ± 2.2		93	127.7 ± 2.0		165	122.5 ± 1.5				
		AA	0	—		0	—		0	—				
	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	—				
	3730G>A	GG	1302	118.4 ± 0.5		.067	1451		126.7 ± 0.5	.714		2753	122.6 ± 0.4	.129
		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	
<i>GGCX</i>	412G>A	GG	1138	118.6 ± 0.5	.317	1295	126.5 ± 0.5	.292	2433	122.5 ± 0.4	.971			
		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				
	8016G>A (Arg325Gln)	GG	730	118.7 ± 0.7		.932	881		125.4 ± 0.6	.996		1611	122.1 ± 0.5	.882
		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	
	8445C>T	CC	1360	118.8 ± 0.5		.715	1578		126.2 ± 0.5	.002		2938	122.5 ± 0.4	.069
		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	
<i>CALU</i>	11G>A (Arg4Gln)	GG	945	118.5 ± 0.6	.537	1125	126.6 ± 0.6	.100	2070	122.6 ± 0.4	.301			
		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				
	344G>A	GG	1282	118.4 ± 0.5		.595	1470		126.3 ± 0.5	.752		2752	122.4 ± 0.4	.722
		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	
	20943T>A	TT	1351	118.8 ± 0.5		.264	1558		126.5 ± 0.5	.342		2909	122.7 ± 0.4	.132
		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\*

Gene	SNP	Genotype	Men			Women			Total					
			n	Protein S Activity, %	P	n	Protein S Activity, %	P	n	Protein S Activity, %	P			
<i>VKORC1</i>	523G>A	GG	825	93.0 ± 0.7	.172	948	83.2 ± 0.6	.407	1773	88.0 ± 0.4	.122			
		GA	40	88.7 ± 3.1		50	81.2 ± 2.4		90	84.9 ± 2.0				
		AA	0	—		0	—		0	—				
	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	—				
	3730G>A	GG	740	93.2 ± 0.7		.786	827		83.6 ± 0.6	.918		1567	88.3 ± 0.5	.832
		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				
<i>GGCX</i>	412G>A	GG	634	92.6 ± 0.8	.196	730	82.8 ± 0.6	.023	1364	87.6 ± 0.5	.016			
		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				
	8016G>A (Arg325Gln)	GG	436	92.4 ± 0.9		.717	500		82.4 ± 0.8	.246		936	87.4 ± 0.6	.234
		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				
	8445C>T	CC	774	93.2 ± 0.7		.581	886		83.2 ± 0.6	.142		1660	88.1 ± 0.5	.282
		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				
<i>CALU</i>	11G>A (Arg4Gln)	GG	545	93.0 ± 0.8	.085	647	82.9 ± 0.7	.740	1192	87.8 ± 0.5	.270			
		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				
	344G>A	GG	733	92.7 ± 0.7		.712	829		83.4 ± 0.6	.350		1562	88.0 ± 0.5	.490
		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				
	20943T>A	TT	761	92.5 ± 0.7		.741	873		83.7 ± 0.6	.507		1634	88.1 ± 0.5	.844
		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		Women		Total	
		Protein C Activity, %	n	Protein C Activity, %	n	Protein C Activity, %	n
<i>PROC</i> , -1657C>T (rs1799808)	CC	118.4 ± 1.5	154	124.5 ± 1.4	181	121.5 ± 1.0	335
	CT	118.5 ± 0.7	690	126.4 ± 0.7	734	122.5 ± 0.5	1424
	TT	118.8 ± 0.7	679	126.9 ± 0.6	844	123.0 ± 0.5	1523
		<i>P</i> = .753		<i>P</i> = .150		<i>P</i> = .185	
<i>PROC</i> , -1644G>A (rs1799809)	GG	116.2 ± 4.8	16	122.4 ± 4.0	22	119.1 ± 3.1	38
	GA	116.6 ± 1.1	321	123.5 ± 1.0	340	120.0 ± 0.7	661
	AA	119.3 ± 0.6	1183	127.3 ± 0.5	1394	123.4 ± 0.4	2577
		<i>P</i> = .025		<i>P</i> = .001		<i>P</i> < .001	
<i>NQO1</i> , 2515C>T (rs1437135)	CC	118.8 ± 1.3	202	125.5 ± 1.1	268	122.1 ± 0.9	470
	CT	118.7 ± 0.7	751	126.9 ± 0.7	833	122.9 ± 0.5	1584
	TT	118.6 ± 0.8	572	126.3 ± 0.7	665	122.5 ± 0.5	1237
		<i>P</i> = .890		<i>P</i> = .745		<i>P</i> = .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	<i>P</i>
Model with <i>GGCX</i> 8016G>A genotype ( <i>R</i> <sup>2</sup> = 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
<i>GGCX</i> 8016G>A genotype	4.46	.006
Model with <i>PROC</i> -1644G>A genotype ( <i>R</i> <sup>2</sup> = 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
<i>PROC</i> -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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REGULAR ARTICLE

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

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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.

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DVT for genetic variation. By genotyping three polymorphisms (–202G>A, 2487A>T, 2729A>G) and two missense mutations (R385K, D468Y) in a Japanese general population, we assessed the prevalence of these genetic variations. We then evaluated the association of sTM levels with genetic variations. We finally compared the genotype prevalence of these genetic variations in DVT patients with those in population-based controls to test whether these mutations are associated with DVT in the Japanese.

## Patients and methods

### DVT patients

A total of 118 Japanese DVT patients (59 men and 59 women, mean age:  $52.3 \pm 16.1$  years old) were recruited from Osaka University Hospital from 2000 to 2004 and the National Cardiovascular Center from 2002 to 2004. All patients examined in this study were unselected patients diagnosed with DVT. Clinical diagnosis of DVT was confirmed by imaging analysis including computerized tomography and ultrasonography.

### Screening of genetic variations in TM gene

Blood samples were obtained from DVT patients and genomic DNA was isolated from peripheral blood leukocytes [17]. All the putative promoter, exon, and 3'-UTR regions in 118 Japanese DVT patients were directly sequenced with an ABI

PRISM3700DNA analyzer (Applied Biosystems, Foster City, CA) using seven sets of primers. Primer sequences are available upon request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [18]. The A of ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [19]. The nucleotide sequence (GenBank Accession ID: AF-495471) was used as a reference sequence.

### General population (Suita Study)

The sample selection and study design of the Suita Study have been described previously [20–22]. Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups, underwent a routine blood examination that included lipid profiles and glucose levels, and underwent blood pressure measurements. The basic characteristics of the individuals have been reported previously [23,24]. sTM levels of 2247 population-based samples were measured by an enzyme-linked immunosorbent assay (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

### Genotyping of mutations and single nucleotide polymorphisms (SNPs) in the general population

Two common SNPs with a minor allele frequency of greater than 5% and all of the missense mutations we detected were tried for genotyping by the

**Table 1** Clinical profiles of 118 DVT patients

Clinical profiles		Clinical profiles	
Age, years $\pm$ S.D.	52.3 $\pm$ 16.1	Nephrotic syndrome, <i>n</i> (%)	0 (0.0)
Women, <i>n</i> (%)	59 (50.0)	Chronic heart failure, <i>n</i> (%)	17 (14.4)
BMI, kg/m <sup>2</sup> , mean $\pm$ S.D.	23.7 $\pm$ 3.2	Diabetes Mellitus, <i>n</i> (%)	47 (39.8)
DVT family history, <i>n</i> (%)	8 (6.8)	Hyperlipidemia, <i>n</i> (%)	48 (40.7)
Previous DVT, <i>n</i> (%)	12 (10.2)	Autoimmune disease, <i>n</i> (%)	11 (9.3)
Pregnancy, <i>n</i> (%)	5 (4.2)	Inflammatory bowel disease, <i>n</i> (%)	2 (1.7)
Stroke, <i>n</i> (%)	1 (1.5)	Estrogen use, <i>n</i> (%)	3 (2.5)
Prolonged immobility, <i>n</i> (%)	14 (11.9)	Steroid use, <i>n</i> (%)	9 (7.6)
Malignancy, <i>n</i> (%)	16 (13.6)	Paralysis, <i>n</i> (%)	5 (4.2)
Major surgery (abd, hip, leg), <i>n</i> (%)	21 (17.8)	Myeloproliferative disease, <i>n</i> (%)	1 (0.8)
Trauma (pelvis, hip, leg), <i>n</i> (%)	3 (2.5)	Reduced plasminogen activity, <i>n</i> (%)	7 (5.9)
Stasis due to compression, <i>n</i> (%)	6 (5.1)	Reduced antithrombin activity, <i>n</i> (%)	7 (5.9)
Central venous catheter, <i>n</i> (%)	0 (0.0)	Reduced protein C activity, <i>n</i> (%)	8 (6.8)
		Reduced protein S antigen, <i>n</i> (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin, ACLb2), <i>n</i> (%)	3 (11.0)

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose  $\geq 126$  mg/dl or non-fasting plasma glucose  $\geq 200$  mg/dl or HbA1c  $\geq 6.5\%$  or use of antidiabetic medication; Hypertension, systolic blood pressure  $\geq 140$  mm Hg and/or diastolic blood pressure  $\geq 90$  mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol  $\geq 220$  mg/dl or use of antihyperlipidemia medication; Myeloproliferative disease, Plt.  $>5 \times 10^5$  and Ht.  $>55\%$ ; Reduced plasminogen activity, plasminogen activity  $<70\%$ ; Reduced antithrombin activity, antithrombin activity  $<80\%$ ; Reduced protein C activity, protein C activity  $<70\%$ ; Reduced protein S antigen, protein S antigen  $<60\%$ .