WILEY InterScience® DISCOVER SOMETHING GREAT

Research article

Molecular basis of antithrombin deficiency in four Japanese patients with antithrombin gene abnormalities including two novel mutations

Mayu Kyotani,[1] Kaoru Okumura,[1] Akira Takagi,[1,2] Takashi Murate,[1,2] Koji Yamamoto,[3] Tadashi Matsushita,[4] Motoi Sugimura,[5] Naohiro Kanayama,[5] Takao Kobayashi,[6] Hidehiko Saito,[7] and Tetsuhito Kojima[1,2]*

[1] Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan
[2] Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan
[3] Division of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan
[4] Department of Hematology-Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[5] Maternal-Fetal Neonatal Care Center, Hamamatsu University School of Medicine, Hamamatsu, Japan
[6] Department of Family and Child Nursing, and Midwifery, Shinshu University School of Health Sciences, Matsumoto, Japan
[7] JR Tokai Genaral Hospital, Nagoya, Japan

We analyzed the antithrombin (*AT*) gene in four unrelated Japanese patients with an AT deficiency, and individually identified four distinct mutations in the heterozygous state. There were two novel mutations, 2417delT leading to a frameshift with a premature termination at amino acid −3 (FS-3Stop) and C2640T resulting in a missense mutation (Ala59Val). Previously reported mutations, T5342C (Ser116Pro) and T72C (Met-32Thr), were also found in the other two patients. To understand the molecular basis responsible for the AT deficiency in these patients, in vitro expression experiments were performed using HEK293 cells transfected with either wild type or respective mutant AT expression vector. We found that −3Stop-AT and −32Thr-AT were not secreted into the culture media, whereas 116Pro-AT and 59Val-AT were secreted normally. We further studied the heparin cofactor activity and the binding to heparin of each recombinant AT molecule. Ser116Pro mutation significantly impaired the binding affinity to heparin resulting in a reduced heparin cofactor activity. In contrast, we found that Ala59Val mutant AT unexpectedly showed a normal affinity to heparin, but severely impaired the heparin cofactor activity. Our findings suggested that FS-3Stop and Met-32Thr mutations are responsible for type I AT deficiency, whereas Ser116Pro and Ala59Val mutations contribute to type II AT deficiency, confirming that there were diverse molecular mechanisms of AT deficiency depend upon discrete *AT* gene abnormalities as reported previously. Am. J. Hematol. 82:702–705, 2007. © 2007 Wiley-Liss, Inc.

Introduction

Antithrombin (AT) is a plasma serine protease inhibitor that inhibits thrombin as well as other activated serine proteases of the coagulation system [1]. Human *AT* gene measures 13.5 kb in length and comprises 7 exons and 6 introns, and is located on human chromosome 1 at q23.1-23.9 [2]. Plasma AT is synthesized by hepatocytes as a 464 amino acid precursor with a 32 amino acid signal peptide, which is cleaved off before secretion of a 432 amino acid mature inhibitor into plasma. It is a single chain glycoprotein with a molecular weight of approximately 58 kDa, which has two important functional domains; one is the reactive site domain located in the C-terminal to inhibit clotting proteases and the other is the heparin binding site domain composed of basic amino acid residues such as 11Lys, 13Arg, 46Arg, 47Arg, 114Lys, 125Lys, and 129Arg in the N-terminal [3].

The inhibition of thrombin by AT, forming a covalent complex in a 1:1 molar ratio, is relatively slow, but is dramatically enhanced in the presence of glycosaminoglycan, heparin [4]. The plasma AT plays a key role in the natural hemostatic balance to maintain blood fluidity, and patients with AT deficiency are susceptible to thromboembolic diseases, particularly deep vein thrombosis of lower limb and pulmonary embolism [5]. Congenital AT deficiency is usually heterozygous and classified into two types: quantitative deficiency (type I) and qualitative deficiency (type II). The latter includes reactive site defect, heparin binding site defect, and pleiotrophic effect AT deficiencies [6].

To date, 172 distinct *AT* gene abnormalities were reported at the Human Gene Mutation Database Web site (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1). In this study, we investigated the nature of AT deficiency in four Japanese female patients with thrombotic complications during pregnancy, and identified four distinct mutations including two novels in the *AT* gene, which might be responsible for discrete phenotypes of AT deficiency.

Contract grant sponsors: Ministry of Health, Labor and Welfare (Japan), Grant-in-Aid for Research on Measures for Intractable Diseases.

*Correspondence to: Tetsuhito Kojima, Department of Medical Technology, Nagaya University School of Health Sciences, 1-1-20 Daiko-Minami, Higashi-ku, Nagoya 461-8673, Japan. E-mail: kojima@met.nagoya-u.ac.jp

M.K. and K.O. contributed equally to this work.

Received for publication 11 July 2006; Revised 19 January 2007; Accepted 23 January 2007

Am. J. Hematol. 82:702–705, 2007.

Published online 9 May 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.20924

TABLE I. Gene Abnormalities and Clinical Features of the Patients with AT Deficiency

Case	Age (years)	Sex	Mutation	Predicted AA change	Location	AT:Ac (%)	AT:Ag (%)
1	33	F	T5342C[a]	Ser116Pro[a]	Exon 3a	31.0	72.4
2	28	F	T72C	Met-32Thr	Exon 1	65.0	61.4
3	24	F	2417delT	FS-3Stop	Exon 2	44.3	ND
4	40	F	C2640T	Ala59Val	Exon 2	62.5	93.2

[a]cDNA and amino acid numbers are according to Old et al. (2).
M, male; F, female; ND, not done; underline, novel mutation.

TABLE II. Oligonucleotide Primers for PCR Amplification of the *AT* Gene

Exon	Sense (5′→3′)	Antisense (5′→3′)	Product (bp)	Annealing (°C)
1	GGAGTGTGGGCAAGAGAG	GAGGTCACAAAACCCAGTAG	366	60
2	TGGGCAGTGGGGCTAGG	ACAAGGTGGCTGGGCAGA	459	65
3a	TAACTAGGCAGCCCACCAAA	TCGGAGGTCAGGGGTAACA	297	60
3b	CTGCCTGGGAAAATGGAGAA	GGGTAAGCTGAAGAGCAAGA	264	55
4	GAATGTTTGTGTTCTTACTTT	AGACTACCTTGCGGGTG	475	55
5	ATGTTTCAAAAAGCCCCAAAG	CACCATGAAGGTTTTGGAGAG	322	57
6	CGGCAGAGTGGCTAATTTAGT	TGTCTTCATTTCAAATGCAGA	335	57

Materials and Methods

Patients and sample preparations

All patients suffered from either deep vein thrombosis or pulmonary embolism during pregnancy and were thus investigated for AT deficiency on the basis of thrombosis. There was no relative with thrombotic disease in all cases. The AT activity and antigen levels of those patients are shown in Table I. Ethical approval for the study was obtained from the Ethics Review Committee of Nagoya University School of Medicine. After obtaining informed consent from all patients, blood samples from the patients, family members, and healthy volunteers were collected in a 1/10 volume of 3.13% sodium citrate. Plasma was separated by centrifugation at × 2000*g* for 20 min, and genomic DNA was isolated from peripheral blood leukocytes as previously described [7].

Identification of gene abnormalities of the patients' *AT* gene

All 7 exons including splice junctions of the *AT* gene were amplified by (PCR) using the primer sets listed in Table II. The PCR amplifications and Cycle sequencing of all PCR products were performed as described previously [8].

Construction of recombinant AT expression vectors

A full length cDNA of human AT was amplified from the human liver cDNA library (Clontech, CA) by PCR, using a primer set (5′-GAAGAT TAGCGGCCATGT-3′ and 5′-GGAAGAGGTGAAAGAATAAG-3′), and used to prepare the wild type human AT expression vector using the full length AT cDNA in pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA) (pcDNA/WT-AT). Subsequently, we prepared the mutant AT expression vectors (pcDNA/116Pro-AT, pcDNA/-32Thr-AT, pcDNA/FS-3Stop-AT, and pcDNA/59Val-AT) by the recombinant PCR method described elsewhere [9].

Transient expression of the recombinant ATs

Human embryo kidney 293 (HEK293) cells were grown in Dulbeco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2. The cells were cultured in 60-mm dishes until they became 50% confluent, and then were transiently transfected with 10 μg of the expression plasmid vectors using the calcium phosphate method as described previously [10]. After incubation in FCS-free DMEM for 24 hr, the cell culture media and cell lysates that had dissolved in reporter lysis buffer (Promega, Madison, USA) were collected, centrifuged at × 1500*g* for 10 min, and prepared as Western blot samples. The Western blot analysis was performed as described previously [11], using a polyclonal anti-AT antibody (rabbit IgG) and a peroxidase-labeled anti-rabbit IgG antibody (Behringwerke AG, Marburg, W. Germany).

Specific activity measurements of recombinant ATs

We transfected HEK293 cells with 20 μg of the recombinant AT expression vectors (pcDNA/WT-AT, pcDNA/116Pro-AT, and pcDNA/59Val-AT) using the calcium phosphate method as described earlier. The transformed cells were selected in culture medium containing 700 μg/ml G418 (Gibco BRL, Rockville, USA), and the stable transformants highly expressing respective recombinant AT molecules were obtained. The antigen levels of recombinant AT molecules were measured by an enzyme-linked immunosorbent assay (ELISA) using a polyclonal sheep anti-AT IgG antibody (Biogenesis England, UK) for capturing, and amplification and detection of the signals were achieved with biotinylated polyclonal sheep anti-AT IgG antibody and avidin peroxidase using ECL Protein Biotinylation Module kit (Amersham Biosciences) according to the manufacturer's protocol. The culture media of the stably transformed cells were concentrated to 2 μg/ml of AT using Centrisart I (Sartorius, Goettingen, Germany), and the AT activities were measured using the N-test AT III-S kit (Nittobo, Tokyo, Japan). The progress AT activity was measured in the absence of heparin. The specific activity of each recombinant AT was calculated as a percentage of the wild type AT activity.

Affinity chromatography of recombinant ATs on heparin-sepharose

Affinity chromatography of each recombinant AT molecule was performed on a heparin–sepharose column. Concentrated media (2 μg/ml of AT) containing wild type-, 116Pro-, or 59Val-AT were subjected to column chromatography on the heparin–sepharose CL6B column (0.8 cm × 0.5 cm) that was equilibrated with 50 mmol/l Tris-HCl buffer (pH7.4) containing 0.01 mol/l citric acid. The column was washed with three-column volumes of the equilibration buffer, and the bound AT was then eluted by a stepwise increase in the concentration of NaCl (0.25, 0.5, 0.75, 1.0, and 1.5 mol/l). The concentration of AT in each fraction was determined by ELISA as described earlier.

Results and Discussion

We analyzed the *AT* gene in four unrelated Japanese patients with an AT deficiency by polymerase chain reaction (PCR) mediated direct sequencing, and identified four distinct mutations individually (Table I). They included two novel mutations (2417delT and C2640T) and two previously reported ones (T5342C and T72C) [12,13], and were all in the heterozygous state. We also detected each mutation in the baby of both cases with the mutation that had been reported before (data not shown). We were not able to obtain any sample from relatives of the cases who had a novel mutation.

We identified two mutations resulting in an abnormality in the signal peptide, the novel 2417delT (FS-3Stop) mutation and the previously reported T72C (Met-32Thr) mutation.

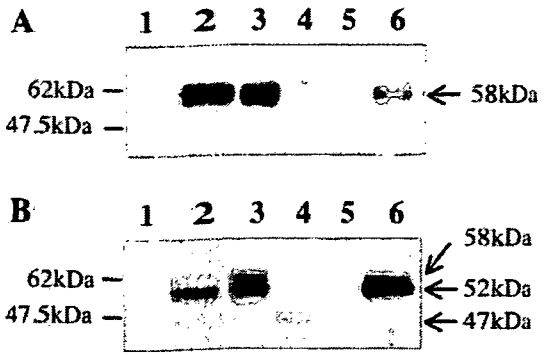

Figure 1. Western blot analysis of the recombinant AT molecules. Wild type and mutant ATs were transiently expressed in HEK293 cells, and the culture media and cell lysates were analyzed by Western blotting. We loaded 10 μg of each sample normalized for total protein. (A) Culture media, (B) cell lysates. Lane1, vector; lane 2, wild type-AT; lane 3, 116Pro-AT; lane 4, -32Thr-AT; lane 5, FS-3Stop-AT; lane 6, 59Val-AT.

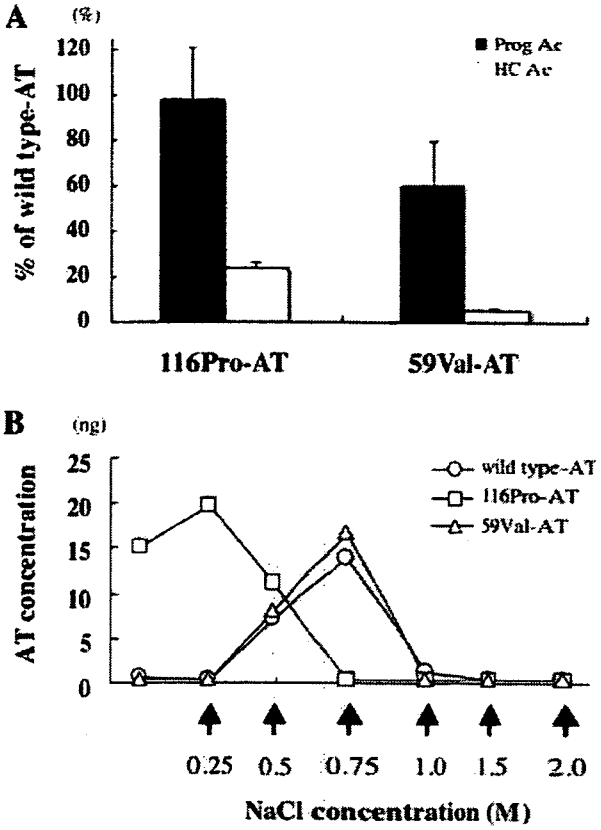

Figure 2. Characteristics of the recombinant AT molecules. (A) Progressive AT activities (Prog Ac: solid column) and heparin cofactor AT activities (Hc Ac: open column) of the recombinant proteins stably expressed in the culture media were examined as described in the Materials and Methods. Every specific AT activities were calculated as a percentage of wild type-AT activity (mean ± SD %, $n = 3$). (B) Affinity chromatographies of the recombinant AT molecules were performed on a heparin–sepharose column as described in the Materials and Methods. The bound AT was eluted by a stepwise increase in the concentration of NaCl (0.25, 0.5, 0.75, 1.0, and 1.5 mol/l), and the concentration of AT in each fraction was determined by ELISA. We applied 100 ng (WT and A59V) or 150 ng (S116P) of the respective recombinant ATs to the heparin column, and eluted 21.4 ng (WT), 24.3 ng (A59V), and 30.3 ng (S116P), respectively. Some of ATs (17.5 ng of WT, 19.5 ng of A59V, and 37.9 ng of S116P) run through the column.

The 2417delT results in a frameshift mutation (FS-3Stop), which encodes 13 abnormal amino acids from the −16Tyr residue in the signal peptide of plasma AT precursor. The FS-3Stop mutant AT could not be secreted into the media, since it was likely to be truncated in the signal peptide. Indeed, we observed that a Western blot analysis for the culture media and cell lysate samples from FS-3Stop-AT expressed cells showed no signal of normal translated protein bands (58 and 52 kDa) (Fig. 1A), but we did detect a considerable signal of a 47 kDa band, which was expected to be an alternative translation initiated from 17Met [14] (Fig. 1B). We also observed a similar result in the samples from −32Thr-AT expressed cells, which is consistent with the previous report [13]. These data suggested that both mutations would likely cause a type I AT deficiency in such patients.

AT has two important functional domains, one is to inhibit clotting protease while the other is to bind heparin, by which the inhibition of protease can be accelerated by ~1000 fold [4]. In the case of thrombin, heparin accelerates the protease inhibitor activity of AT via both approximation effects and conformational changes, while in the case of factor Xa and other clotting proteases, it enough activates via only conformational changes [15]. The reactive site and the heparin binding site are located in the C-terminal and in the N-terminal of the AT molecule, respectively. We identified two missense mutations in the *AT* gene, which were previously reported as Ser116Pro mutation and a novel Ala59Val mutation. Both mutations are located in the N-terminal, or so-called heparin binding domain. Okajima et al. reported that the patient plasma AT bearing the Ser116Pro mutation to have no affinity for heparin and this mutation was thus thought to be causative for type II AT deficiency [12]. We observed consistent results, which included a normal progressive AT activity (98.3% ± 22.6% of the wild type-AT), an impairment of the heparin binding affinity and a significantly reduced specific heparin cofactor activity in the recombinant 116Pro-AT molecule (23.9% ± 2.5%) (Fig. 2A). We speculate that the recombinant 116P-AT may have some differences in conformation from natural mutant AT III Nagasaki, so that it might show less impaired activity (23.9% normal versus essentially inactive). Indeed, heparin affinity of the recombinant 116P-AT seemed to be also less impaired than that of AT III Nagasaki reported by others [12] (Fig. 2B). In contrast, the recombinant 59Val-AT also abolished the specific heparin cofactor activity (5.2% ± 0.2%), but surprisingly showed a normal affinity to heparin–sepharose as well as a substantially normal progressive AT activity (60.8% ± 16.3%) (see Fig. 2). Similar results were obtained from the Case 4 patient's plasma sample, which showed normal affinity of the patient's AT to the heparin–sepharose, but its AT activity was reduced to about half of the normal plasma regardless of addition of heparin (data not shown). The 59Ala locates at center of α-helix consisting of residues 47Arg to 69Ser, via which heparin pentasaccharide primarily interacts with AT [16]. Therefore, it might be possible that the Ala59Val

mutation makes a great influence to the AT conformational status, resulting in an impaired expulsion of the reactive center loop to free the P1 393Arg for the interaction of the target proteases, even if its heparin-binding is normal. As a result, the Ala59Val mutation would be the cause of type II AT deficiency in the patient.

In conclusion, we investigated the molecular basis of AT deficiency in four Japanese female patients with thrombotic complications during pregnancy, and identified four distinct causative mutations including two novel mutations in their *AT* gene. Our findings confirmed that there were diverse molecular mechanisms of AT deficiency depend upon discrete *AT* gene abnormalities as reported previously.

Acknowledgments

The authors thank C. Wakamatsu for her expert technical assistance and are also indebted to M. Yamashita, N. Yamakage, M. Ikejiri, T. Yamada and H. Okada for their helpful discussions.

References

1. Rosenberg RD. Biochemistry of heparin antithrombin interactions, and the physiologic role of this natural anticoagulant mechanism. Am J Med 1989;87:2S–9S.
2. Olds RJ, Lane DA, Chowdhury V, De Stefano V, Leone G, Thein SL. Complete nucleotide sequence of the antithrombin gene: Evidence for homologous recombination causing thrombophilia. Biochemistry 1993;32:4216–4224.
3. Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. The anticoagulant activation of antithrombin by heparin. Proc Natl Acad Sci USA 1997;94:14683–14688.
4. Olds RJ, Lane DA, Mille B, Chowdhury V, Thein SL. Antithrombin: The principal inhibitor of thrombin. Semin Thromb Hemost 1994;20:353–372.
5. Bauer KA, Rosenberg RD. Congenital antithrombin III deficiency: Insights into the pathogenesis of the hypercoagulable state and its management using markers of hemostatic system activation. Am J Med 1989;87:39S–43S.
6. Lane DA, Olds RJ, Conard J, et al. Pleiotropic effects of antithrombin strand 1C substitution mutations. J Clin Invest 1992;90:2422–2433.
7. Toyozumi H, Kojima T, Matsushita T, Hamaguchi M, Tanimoto M, Saito H. Diagnosis of hemophilia B carriers using two novel dinucleotide polymorphisms and Hha I RFLP of the factor IX gene in Japanese subjects. Thromb Haemost 1995;74:1009–1014.
8. Tsukahara A, Yamada T, Takagi A, et al. Compound heterozygosity for two novel mutations in a severe factor XI deficiency. Am J Hematol 2003;73:279–284.
9. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989; 77:51–59.
10. Sobue S, Hagiwara K, Banno Y, et al. Transcription factor specificity protein 1 (Sp1) is the main regulator of nerve growth factor-induced sphingosine kinase 1 gene expression of the rat pheochromocytoma cell line, PC12. J Neurochem 2005;95:940–949.
11. Yamada T, Takagi A, Takeshita K, et al. Enzyme immunoassay for measurement of murine plasminogen activator inhibitor-1, employing a specific antibody produced by the DNA vaccine method. Thromb Res 2003;111:285–291.
12. Okajima K, Abe H, Maeda S, et al. Antithrombin III Nagasaki (Ser116-Pro): A heterozygous variant with defective heparin binding associated with thrombosis. Blood 1993;81:1300–1305.
13. Nagaizumi K, Inaba H, Amano K, et al. Five novel and four recurrent point mutations in the antithrombin gene causing venous thrombosis. Int J Hematol 2003;78:79–83.
14. Fitches AC, Appleby R, Lane DA, De Stefano V, Leone G, Olds RJ. Impaired cotranslational processing as a mechanism for type I antithrombin deficiency. Blood 1998;92:4671–4676.
15. Quinsey NS, Greedy AL, Bottomley SP, Whisstock JC, Pike RN. Antithrombin: In control of coagulation. Int J Biochem Cell Biol 2004;36:386–389.
16. Li W, Johnson DJ, Esmon CT, Huntington JA. Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. Nat Struct Mol Biol 2004;11:857–862.

An Sp1 binding site mutation of the *PROS1* promoter in a patient with protein S deficiency

Protein S (PS) is a vitamin K-dependent plasma glycoprotein which functions as an important natural anticoagulant, and its deficiency is known to be one of the major risk factors for thrombosis. PS serves as a cofactor in the protein C (PC) pathway for activated PC, which regulates blood coagulation by inactivating factors Va and VIIIa (Dahlbäck, 1991) and also directly inhibits factors Va (Heeb *et al*, 1993) and Xa (Heeb *et al*, 1994) independently of PC. Although many PS (alpha) gene (*PROS1*) abnormalities associated with PS deficiency have been identified, no mutation has been reported in the *PROS1* promoter region to date. This paper describes the first report of a *PROS1* promoter mutation responsible for PS deficiency. The mutation, a C-to-T substitution, was within the core binding site of transcription factor Sp1 and disrupted binding of Sp1 to the *PROS1* promoter, thereby causing the reduced *PROS1* expression in reporter gene assays.

The patient was a 32-year-old Japanese male with portal vein thrombosis who had started on anticoagulation therapy with heparin and warfarin. PC decreases more rapidly by warfarisation than PS; however, this patients' plasma levels of free and total PS antigens were notably low (31% and 68% respectively), while that of PC antigen was normal (128%), on the seventh day of the initial low-dose warfarisation. Therefore, he was diagnosed with congenital PS deficiency.

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine. DNA samples were obtained with informed consent in accordance with the Declaration of Helsinki. Polymerase chain reaction (PCR)-based sequence analysis of *PROS1* was performed as described previously (Okada *et al*, 2004). Sequencing of the 5′-flanking region of the patient's *PROS1* demonstrated a C-to-T substitution at position 168-bp upstream from the translational start site (-168 C → T). *PmlI* PCR-restriction fragment length polymorphism analysis using a partially mismatched sense primer (5′-CCCTCTCGGGCT-GGGCGCTGGGAGCacG-3′) revealed that the -168 C → T was not a polymorphism (data not shown).

To investigate, whether -168 C → T influenced *PROS1* promoter function, we analysed the wild-type and mutant promoter activity by luciferase reporter assay in the transiently transfected HepG2 cells. The immediate 5′-flanking region of the wild-type or mutant *PROS1* promoter was amplified by PCR and cloned into the pGL3-basic-derived vector (Kokame *et al*, 2001). The 5-μg luciferase vector and 1-μg pSV-β-galactosidase plasmid (Promega Corp, Madison, WI, USA) were cotransfected to HepG2 cells by the calcium precipitation method. Luciferase and β-galactosidase activities were measured at 24 h after glycerol shock, and the relative promoter activity was represented as the luciferase/β-galactosidase activity. The mean value of the relative luciferase activity with the mutant plasmid was 23·3 ± 12·9% ($n = 9$) of that with the wild-type. Thus, a fragment of the mutant promoter exhibited impaired activity compared with the corresponding wild-type promoter.

The -168 C → T mutation occurs within a putative Sp1 binding site. Sp1 is a versatile protein regulating the expression of many different genes. Binding sites for Sp1 are widely distributed in TATA-less GC-rich promoters (Dynan, 1986) and have been proposed to functionally compensate for the absence of canonical TATA box (Pugh & Tjian, 1991). Therefore, we evaluated the behaviour of a 20-bp *PROS1* promoter fragment (from -176 to -157) containing this mutation in interaction with nuclear extracts from HepG2 cells by electrophoretic mobility shift assay (EMSA) (Fig 1). EMSA was performed with 5-μg nuclear extract from HepG2 cells and biotin-labeled double-strand DNA probes (600 fmol) using a LightShift™ Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). In the supershift experiment, nuclear extract was incubated with biotin-labeled probes and anti-Sp1 antibody (sc59X) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Biotin-labeled DNA probes were transferred to Hybond-N membrane (Amersham Bioscience, Piscataway, NJ, USA) and analysed by the Light Capture gel/documentation system (Atto, Tokyo, Japan). The wild-type oligonucleotide was found to bind a protein present in HepG2 nuclear extract, but the mutant showed a decreased nuclear protein binding. In the bound HepG2 nuclear proteins, two complexes (Fig 1, arrows) were specific, as they could be competed away by incubation with increasing amounts of unlabeled wild-type oligonucleotide, but not by equivalent amounts of unlabeled mutant. Thus, the wild-type oligonucleotide served as the stronger competitor as opposed to the mutant. The presence of the ubiquitous transcription factor Sp1 in the slowest-mobility complex (Fig 1, *) was shown by the supershift experiment with anti-Sp1 antibody, indicating that the wild-type oligonucleotide of the human *PROS1* promoter sequence contains an actual Sp1 binding site.

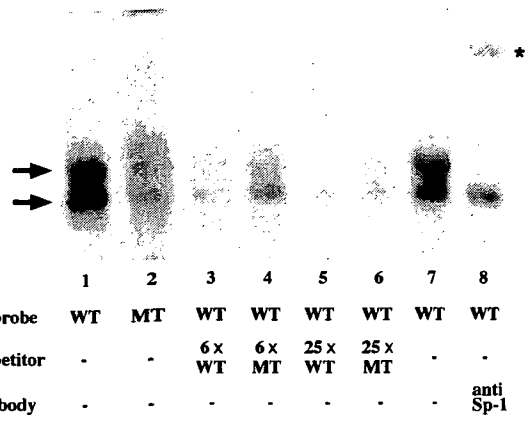

Fig 1. Electrophoretic mobility shift assay using wild-type and mutant oligonucleotide sequences. The -168 C-to-T mutation (-168 C → T) diminished binding of Sp1 to the *PROS1* promoter sequence. A biotin-labeled wild-type (WT) oligonucleotide encompassing the Sp1 binding site (from -176 to -157 prior to the translation start site) in the human *PROS1* promoter region showed binding to nuclear extract proteins (arrows) from HepG2 cells (lanes 1 and 7). A biotin-labeled mutant (MT) oligonucleotide showed decreased signals of protein combinations (lane 2). Reactions were performed with incubation of unlabeled competitor oligonucleotides at 6× concentrations of WT and MT sequence (lanes 3 and 4 respectively), and 25× concentrations of WT and MT sequence (lanes 5 and 6 respectively). Sp1-specific antibody was added to the reaction, resulting in a supershift of the antibody-protein-oligonucleotide probe complex (lane 8, *).

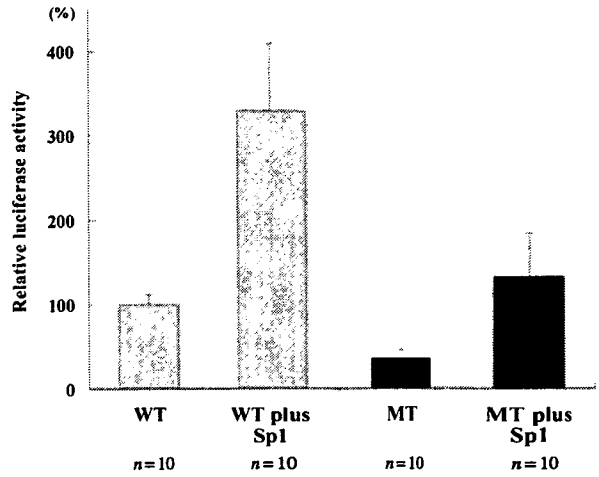

Fig 2. Cotransfection of an Sp1 expression vector alters expression of reporter gene from vectors with wild-type and -168 C → T mutant *PROS1* promoter sequence. wild-type (WT) or mutant (-168 C → T; MT) reporter vector was transiently transfected into HepG2 cells with or without Sp1 expression vector as described in methods. The results shown (37·0 ± 9·3% for mutant plasmid vs. 100·0 ± 11·3% for wild-type plasmid, in the absence of Sp1 expression vector; 133·4 ± 51·5% for mutant plasmid vs. 328·9 ± 81·1% for wild-type plasmid, in the presence of Sp1 expression vector) were the averages from three experiments. Each of the relative luciferase activities was normalised for transfection efficiency by cotransfection with a β-galactosidase control plasmid and calculated as a percentage of the mean value of wild-type reporter vector without Sp1 expression vector (mean ± SD %). The total number of replicates in each group is shown.

Transcription from the *PROS1* promoter is directed from multiple start sites (de Wolf *et al*, 2005), and recently two groups have characterised essential regulatory elements in the 5′-flanking region of the *PROS1* (Tatewaki *et al*, 2003; de Wolf *et al*, 2006). Various transcription factor binding sites were identified within the first 400-bp proximal to the *PROS1* translational start site, including multiple binding sites for ubiquitous transcription factors Sp1 and Sp3. Further confirmation that the Sp1 binding disruption by this mutation leads to reduced promoter activity is shown in Fig 2. One-microgram of pEVR2/Sp1 vector encoding Sp1 (kindly provided by Dr Suske, Phillipps-Universität Marburg, Marburg, Germany), or pEVR2 without Sp1 cDNA as a negative control vector was used for the cotransfection experiment (16 h after glycerol shock). Cotransfection with Sp1 expression plasmid elevated luciferase expression from both wild-type and mutant reporter vectors, although to a significantly lesser extent with the mutant than with the wild-type plasmid. These data suggested that a -168 C → T mutation would reduce *PROS1* mRNA transcription, because of an impairment of Sp1 binding to the *PROS1* promoter sequence, leading to PS deficiency.

In summary, this is the first report of a *PROS1* promoter mutation (-168 C → T) in the Sp1 binding site of a PS deficient patient that strongly impaired its promoter function.

Naomi Sanda[1]
Yuta Fujimori[1]
Takahiro Kashiwagi[1]
Akira Takagi[1,2]
Takashi Murate[1,2]
Emi Mizutani[3,4]
Tadashi Matsushita[3]
Tomoki Naoe[3]
and Tetsuhito Kojima[1,2]

[1]Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, [2]Department of Medical Technology, Nagoya University School of Health Sciences, [3]Department of Haematology–Oncology, Nagoya University Graduate School of Medicine, Nagoya, and [4]Department of Haematology, Ogaki Municipal Hospital, Ogaki, Japan.
E-mail: kojima@met.nagoya-u.ac.jp

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

We gratefully acknowledge C. Wakamatsu for expert technical assistance, and members of the Kojima's and Murate's laboratories for their helpful discussions. This study was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology (17590490), and the Japanese Ministry of Health, Labour and Welfare (Research on Measures for Intractable Diseases).

References

Dahlbäck, B. (1991) Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. *Thrombosis and Haemostasis*, **66**, 49–61.

Dynan, W.S. (1986) Promoters for housekeeping genes. *Trends in Genetics*, **2**, 196–197.

Heeb, M.J., Mesters, R.M., Tans, G., Rosing, J. & Griffen, J.H. (1993) Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. *The Journal of Biological Chemistry*, **268**, 2872–2877.

Heeb, M.J., Rosing, J., Bakker, H.M., Fernandez, J.A., Tans, G. & Griffin, J.H. (1994) Protein S binds to and inhibits factor Xa. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 2728–2732.

Kokame, K., Kato, H. & Miyata, T. (2001) Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *The Journal of Biological Chemistry*, **276**, 9199–9205.

Okada, H., Takagi, A., Murate, T., Adachi, T., Yamamoto, K., Matsushita, T., Takamatsu, J., Sugita, K., Sugimoto, M., Yoshioka, A., Yamazaki, T., Saito, H. & Kojima, T. (2004) Identification of protein S alpha gene mutations including four novel mutations in eight unrelated patients with protein S deficiency. *British Journal of Haematology*, **126**, 219–225.

Pugh, B.F. & Tjian, R. (1991) Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes and Development*, **5**, 1935–1945.

Tatewaki, H., Tsuda, H., Kanaji, T., Yokoyama, K. & Hamasaki, N. (2003) Characterization of the human protein S gene promoter: a possible role of transcription factors Sp1 and HNF3 in liver. *Thrombosis and Haemostasis*, **90**, 1029–1039.

de Wolf, C.J., Cupers, R.M., Bertina, R.M. & Vos, H.L. (2005) Initiation of protein S mRNA synthesis in human liver and various cell lines. *Journal of Thrombosis and Haemostasis*, **3**, 410–412.

de Wolf, C.J., Cupers, R.M., Bertina, R.M. & Vos, H.L. (2006) The constitutive expression of anticoagulant protein S is regulated through multiple binding sites for Sp1 and Sp3 transcription factors in the protein S gene promoter. *The Journal of Biological Chemistry*, **281**, 17635–17643.

Keywords: protein S deficiency, *PROS1* promoter, Sp1, luciferase assay, electrophoretic mobility shift assay.

First published online 26 June 2007
doi:10.1111/j.1365-2141.2007.06668.x

Distinguishing between non-identical twins: platelet type and type 2B von Willebrand disease

We have been following with interest the recent series of reports dealing with the diagnosis of type 2B von Willebrand disease (VWD) and the closely similar platelet type VWD (PT-VWD) (Enayat *et al*, 2006; Favaloro, 2006; Favaloro, 2007; Whalley & Perry, 2007). Nurden *et al* (2007) reported a recent addition to this forum; a second PT-VWD case with Gly 233 Ser demonstrating once again mis/under diagnosis of PT-VWD. In response to the original invitation by Dr Favaloro to hear of the experience of other co-workers, we would like to contribute with our own experience.

We have two different experiences in this respect: a previous UK experience and a recent Canadian one. Our UK experience (Othman *et al*, 2005) was based on documenting a 27 bp deletion in *GPIBA* in a patient with a longstanding diagnosis of type 2B VWD. During pregnancy, continued bleeding and a dramatic reduction of her platelet count in response to von Willebrand factor/factor VIII (VWF/FVIII) preparations, which improved only on platelet concentrate administration, gave the first alert to the possibility of PT-VWD. In Canada, over the past 3 years we have analysed 14 patients referred to our molecular haemostasis laboratory from various Canadian hemophilia clinics for genetic testing with a phenotypic diagnosis of type 2B VWD. Sequencing of exon 28 of *VWF* revealed sequence variations in eight of these patients. Recently, we analysed the *GPIBA* gene in the remaining six patients and have identified a Gly 233 Val substitution in two of them. No platelet mixing studies or cryoprecipitate challenge was performed in these patients. No family members have been tested, however, one of the two patients had a second degree relative who was diagnosed in a UK centre with PT-VWD and had the same mutation. Although there could be some uncertainty about the phenotypic diagnosis in some of these patients, one can conclude that some PT-VWD cases are being missed among those with type 2B VWD and that the genetic analysis has subsequently confirmed the diagnosis.

Thrombosis Research (2007) 119, 35–43

ELSEVIER

THROMBOSIS RESEARCH

intl.elsevierhealth.com/journals/thre

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*

Received 9 December 2005; received in revised form 19 December 2005; accepted 20 December 2005
Available online 28 February 2006

KEYWORDS
Thrombomodulin;
Deep vein thrombosis;
Gene mutations;
Soluble thrombomodulin;
Japanese;
Case-control study

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.

doi:10.1016/j.thromres.2005.12.012

with that in the age, body mass index-adjusted population-based controls.
Results: We identified one neutral mutation (H381) and three missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$) of TM in the DVT patients. Age-adjusted mean values of sTM were lower in C-allele carriers of 2729A>C than in noncarriers in the Japanese general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$). Additionally, the CC genotype of this mutation was more common in the male DVT patients than in the male individuals of the general population (odds ratio=2.76, 95% confidence interval=1.14–6.67; $p=0.02$). This mutation was in linkage disequilibrium (r-square>0.9) with A455V mutation.
Conclusions: TM mutations, especially those with a haplotype consisting of 2729A>C and A455V missense mutation, affect sTM levels, and may be associated with DVT in Japanese.

Introduction

Family-based studies have established that venous thromboembolism is, at least in part, an inherited disease with estimated heritabilities of approximately 60% [1,2]. The mode of inheritance of venous thromboembolism is probably complex [2]. Moreover, family-based and twin studies have established that over 25 plasma hemostasis-related analytes (traits) both correlate with thrombosis and are heritable [3–5]. In Caucasians, the factor V-Leiden mutation and prothrombin G20210A mutation are widely recognized as genetic risk factors for deep vein thrombosis (DVT) [6]. However these mutations are not present in the Japanese [7,8]. Recently, we and others found that the protein S (PS) K196E mutation, known as the PS Tokushima mutation, is a genetic risk for DVT in the Japanese population, indicating large differences in the genetics of DVT among ethnicities [9,10].

Thrombomodulin (TM) is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells [11]. The anticoagulant function of TM is mediated by interaction with thrombin and protein C (PC). Endothelial membrane-bound TM forms a high-affinity complex with thrombin via thrombin exosite 1, and inhibits thrombin interaction with fibrinogen and protease-activated receptor-1. In contrast, the thrombin–TM complex is a potent activator of PC, and TM enhances thrombin-dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic formation of activated PC (APC) comprise an essential anticoagulant mechanism that prevents the amplification of thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC.

TM encoded by an intron-less gene consists of a large N-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail [12]. The extracellular region is comprised of an N-terminal lectin-like domain followed by six tandem repeats of epidermal growth factor (EGF)-like domains, and a glycosylated (chondroitin sulfate) serine/threonine-rich domain. The thrombin-binding region has been localized to the fifth and sixth EGF-like domains, while the fourth EGF-like domain is required for PC binding to the thrombin–TM complex. The serine/threonine-rich spacer region is required for both thrombin binding and TM cofactor activity for membrane-associated TM. The chondroitin sulfate domain may stabilize thrombin binding to TM, possibly by interacting with the thrombin apolar region [13,14].

Animal model data suggest that TM dysfunction or deficiency is associated with a prothrombotic disorder. Knock-in mice with a TM mutant that has a mutation corresponding to human E387P exhibit a prothrombotic disorder [15]. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble TM (sTM) to catalyze in vitro thrombin activation of PC to APC. Mice with TM deficiency limited to the vascular endothelium die shortly after birth as a result of a consumptive coagulopathy that can be prevented by warfarin anticoagulation [16].

Based on the important antithrombotic role of TM, we hypothesized that genetic variations within the TM gene that alter TM expression and/or impair anticoagulant function could predispose to venous thromboembolism. To test this hypothesis, we screened the promoter, exon, and 3′-untranslated regions (3′-UTR) of the TM gene in unrelated patients with idiopathic, objectively confirmed

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

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication; Hypertension, systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Myeloproliferative disease, Plt. $>5\times10^5$ 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%.

TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r-square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means ± S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t-test or X^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was established at $p<0.05$. Linkage disequilibrium was calculated using SNPAlyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*−832C>A		Promoter		99.6	0.4	gggcagagggcg [c/a] tggtgttaggcc	
*−754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggtttcctga	
*−265C>A		Exon(5′UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
−202G>A	A	Exon(5′UTR)		89.2	10.8	ggagggagggcc [g/a] ggcacttataaa	
*−58G>C		Exon(5′UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gcccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaaggtg [g/t] acggtggcgaca	
1754C>T		Exon(3′UTR)		98.7	1.3	aggagcctggct [c/t] cgtccaggagcc	rs13306852
2005G>A	A	Exon(3′UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggaggg	rs3176134
*2230T>C		Exon(3′UTR)		99.6	0.4	tcttggtgaatt [t/c] ttttttcctagc	
*2487A>T		Exon(3′UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3′UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3′UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521−3522insT		3′flanking		99.6	0.4	ctcgggttgtgt [−/t] gtctgttcactt	
*3559T>A		3′flanking		99.6	0.4	gccctcatttta [t/a] gtcattaaatgg	

LD, mutations in linkage disequilibrium (group A; r-square=0.84, group B r-square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women ($n=1215$)	Men ($n=1032$)	p
Age, years ± S.D.	64.6 ± 10.7	67.1 ± 10.9	<0.0001
Systolic blood pressure, mm Hg ± S.D.	123.5 ± 19.8	126.1 ± 17.9	0.0008
Diastolic blood pressure, mm Hg ± S.D.	74.3 ± 10.4	77.2 ± 10.4	<0.0001
Body mass index, kg/m^2 ± S.D.	22.4 ± 3.2	23.4 ± 3.0	<0.0001
Total cholesterol, mg/dl ± S.D.	215.9 ± 31.6	198.7 ± 31.5	<0.0001
HDL-cholesterol, mg/dl ± S.D.	64.4 ± 15.1	55.2 ± 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure ≧ 140 mm Hg and/or diastolic blood pressure ≧ 90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧ 220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose ≧ 126 mg/dl or non-fasting plasma glucose ≧ 200 mg/dl or HbA1c ≧ 6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's t-test or X^2 analysis.

mutations were missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$). Four mutations within the TM promoter region and the 5′-untranslated region (5′-UTR) (−832C>A, −754G>C, −265C>A, −58G>C) were rare. Twenty-five patients were heterozygous carriers for the −202G>A mutation within the promoter region, which was reported as a −33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (r-square>0.8) with 2005G>A in the 3′-UTR. No patients were carriers for previously reported mutations in the lectin-like domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain ($n=53$ heterozygous, $n=14$ homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (r-square>0.9) with the 2729A>C mutation within the 3′-UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women	Men	Total	Women	Men	Total
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
−202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p
−202 G>A	GG	16.9 ± 1.6		17.0 ± 1.6		19.2 ± 1.9		19.6 ± 1.9	
	GA+AA	17.4 ± 0.2	0.73	17.4 ± 0.2	0.77	19.9 ± 0.2	0.68	19.9 ± 0.2	0.87
1208 G>A	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(R385K)	GA+AA	16.2 ± 2.4	0.62	16.0 ± 2.3	0.54	20.5 ± 2.2	0.79	20.4 ± 2.2	0.84
1456 G>T	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(D468Y)	GT+TT	18.1 ± 1.0	0.51	18.1 ± 1.0	0.52	22.2 ± 1.7	0.20	22.6 ± 1.7	0.11
2487 A>T	AA	17.6 ± 0.2		17.6 ± 0.2		20.0 ± 0.2		20.0 ± 0.2	
	AT+TT	16.7 ± 0.4	0.04	16.7 ± 0.4	0.04	19.6 ± 0.6	0.54	19.5 ± 0.6	0.40
2729 A>C	AA	17.9 ± 0.2		17.9 ± 0.2		20.4 ± 0.3		20.3 ± 0.3	
	AC+CC	16.7 ± 0.3	<0.01	16.8 ± 0.3	<0.01	19.4 ± 0.3	0.03	19.5 ± 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (−202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects. As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p=0.02$ and odds ratio = 2.98, 95% confidence interval = 0.21–7.33; $p=0.02$, respectively) (Table 6). This mutation was in linkage disequilibrium (r-square > 0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3′-UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An in vitro study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17β-estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H_2O_2, or hypochlorous acid generated from H_2O_2 by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3′-UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

Acknowledgements

We would like to thank Ms. Junko Ishikawa of the National Cardiovascular Center for her technical assistance. We also thank Drs. Otosaburo Hishikawa, Yasushi Kotani, Katsuyuki Kawanishi, Toshifumi Mannami, and Mr. Tadashi Fujikawai for their continuous support of our population survey in Suita city. We are grateful to the members of the Satsuki-Junyukai. This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; from the Japan Society for the Promotion of Science; and from the Program for Promotion of Fundamental Studies in National Institute of Biomedical Innovation of Japan.

References

[1] Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, et al. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia. *Am J Hum Genet* 2000;**67**:1452-9.

[2] Heit JA, Phelps MA, Ward SA, Slusser JP, Petterson TM, De Andrade M. Familial segregation of venous thromboembolism. *J Thromb Haemost* 2004;**2**:731-6.

[3] Rosendaal FR, Bovill EG. Heritability of clotting factors and the revival of the prothrombotic state. *Lancet* 2002;**359**:638-9.

[4] de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet* 2001;**357**:101-5.

[5] Ariens RA, de Lange M, Snieder H, Boothby M, Spector TD, Grant PJ. Activation markers of coagulation and fibrinolysis in twins: heritability of the prethrombotic state. *Lancet* 2002;**359**:667-71.

[6] Martinelli I. Risk factors in venous thromboembolism. *Thromb Haemost* 2001;**86**:395-403.

[7] 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.

[8] 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**:451-2.

[9] Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005;**38**:908-15.

[10] Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, et al. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006;**107**:1737-8.

[11] Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci U S A* 1981;**78**:2249-52.

[12] Weiler H, Isermann BH. Thrombomodulin. *J Thromb Haemost* 2003;**1**:1515-24.

[13] Sadler JE, Lentz SR, Sheehan JP, Tsiang M, Wu Q. Structure–function relationships of the thrombin–thrombomodulin interaction. *Haemostasis* 1993;**23(Suppl 1)**:183-93.

[14] Dittman WA, Majerus PW. Structure and function of thrombomodulin: a natural anticoagulant. *Blood* 1990;**75**:329-36.

[15] Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW, Rayburn H, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. *J Clin Invest* 1998;**101**:1983-91.

[16] Isermann B, Hendrickson SB, Zogg M, Wing M, Cummiskey M, Kisanuki YY, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. *J Clin Invest* 2001;**108**:537-46.

[17] Kamide K, Tanaka C, Takiuchi S, Miwa Y, Yoshii M, Horio T, et al. Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004;**27**:333-8.

[18] Okuda T, Fujioka Y, Kamide K, Kawano Y, Goto Y, Yoshimasa Y, 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**:387-94.

[19] Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* 1998;**11**:1-3.

[20] Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study. *Stroke* 1997;**28**:518-25.

[21] 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**:2958-65.

[22] Mannami T, Baba S, Ogata J. Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita Study. *Arch Intern Med* 2000;**160**:2297-303.

[23] Kokubo Y, Kamide K, Inamoto N, Tanaka C, Banno M, Takiuchi S, 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**:507-15.

[24] Kamide K, Kokubo Y, Yang J, Tanaka C, Hanada H, Takiuchi S, et al. Hypertension susceptibility genes on chromosome 2p24-p25 in a general Japanese population. *J Hypertens* 2005;**23**:955-60.

[25] Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003;**26**: 301-6.

[26] Li YH, Chen CH, Yeh PS, Lin HJ, Chang BI, Lin JC, et al. Functional mutation in the promoter region of thrombomodulin gene in relation to carotid atherosclerosis. *Atherosclerosis* 2001;**154**:713-9.

[27] Ohlin AK, Norlund L, Marlar RA. Thrombomodulin gene variations and thromboembolic disease. *Thromb Haemost* 1997;**78**:396-400.

[28] Franchi F, Biguzzi E, Cetin I, Facchetti F, Radaelli T, Bozzo M, et al. Mutations in the thrombomodulin and endothelial protein C receptor genes in women with late fetal loss. *Br J Haematol* 2001;**114**:641-6.

[29] Faioni EM, Merati G, Peyvandi F, Bettini PM, Mannucci PM. The G1456 to T mutation in the thrombomodulin gene is not frequent in patients with venous thrombosis. *Blood* 1997;**89**:1467.

[30] Ohlin AK, Marlar RA. The first mutation identified in the thrombomodulin gene in a 45-year-old man presenting with thromboembolic disease. *Blood* 1995;**85**:330-6.

[31] Kunz G, Ohlin AK, Adami A, Zoller B, Svensson P, Lane DA. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. *Blood* 2002;**99**:3646-53.

[32] Thude H, Wilkens A, Anders O, Barz D. Analysis of the thrombomodulin gene in patients with venous thrombosis. *Thromb Res* 2002;**107**:109-14.

[33] Aleksic N, Folsom AR, Cushman M, Heckbert SR, Tsai MY, Wu KK. Prospective study of the A455V polymorphism in the thrombomodulin gene, plasma thrombomodulin, and incidence of venous thromboembolism: the LITE Study. *J Thromb Haemost* 2003;**1**:88-94.

[34] Heit JA, Petterson TM, Owen WG, Burke JP, De Andrade M, Melton III LJ. Thrombomodulin gene polymorphisms or haplotypes as potential risk factors for venous thromboembolism: a population-based case-control study. *J Thromb Haemost* 2005;**3**:710-7.

[35] Chao TH, Li YH, Chen JH, Wu HL, Shi GY, Tsai WC, et al. Relation of thrombomodulin gene polymorphisms to acute myocardial infarction in patients < or =50 years of age. *Am J Cardiol* 2004;**93**:204-7.

[36] Takahashi Y, Hosaka Y, Imada K, Adachi T, Niina H, Mochizuki H. Species specificity of the anticoagulant activity of human urinary soluble thrombomodulin. *Thromb Res* 1998;**89**:187-97.

[37] Ohlin AK, Larsson K, Hansson M. Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma. *J Thromb Haemost* 2005;**3**:976-82.

[38] Yamada N, Wada H, Nakase T, Minamikawa K, Nagaya S, Nakamura M, et al. Hemostatic abnormalities in patients with pulmonary embolism compared with that in deep vein thrombosis. *Blood Coagul Fibrinolysis* 1995;**6**:627-33.

[39] Trifiletti A, Scamardi R, Pizzoleo MA, Soraci S, Nevoso A, Bagnato L, et al. Haemostatic changes in patients with deep vein thrombosis. *Panminerva Med* 1997;**39**: 21-23.

[40] Smith A, Quarmby JW, Collins M, Lockhart SM, Burnand KG. Changes in the levels of soluble adhesion molecules and coagulation factors in patients with deep vein thrombosis. *Thromb Haemost* 1999;**82**:1593-9.

[41] Mohri M, Suzuki M, Sugimoto E, Sata M, Yamamoto S, Maruyama I. Effects of recombinant human soluble thrombomodulin (rhs-TM) on clot-induced coagulation in human plasma. *Thromb Haemost* 1998;**80**:925-9.

[42] Kearon C, Comp P, Douketis J, Royds R, Yamada K, Gent M. Dose–response study of recombinant human soluble thrombomodulin (ART-123) in the prevention of venous thromboembolism after total hip replacement. *J Thromb Haemost* 2005;**3**:962-8.

[43] Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom H, Juneja H, et al. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. *Lancet* 1999;**353**:1729-34.

[44] Richardson MA, Berg DT, Calnek DS, Ciaccia AV, Joyce DE, Grinnell BW. 17β-estradiol, but not raloxifene, decreases thrombomodulin in the antithrombotic protein C pathway. *Endocrinology* 2000;**141**:3908-11.

[45] Glaser CB, Morser J, Clarke JH, Blasko E, McLean I, Kuhn I, et al. Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation. *J Clin Invest* 1992;**90**:2565-73.

[46] Lentz SR, Tsiang M, Sadler JE. Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and posttranscriptional mechanisms. *Blood* 1991;**77**:542-50.

[47] Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003;**33**:177-82.

International Journal of
HEMATOLOGY

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*

Received December 16, 2005; received in revised form January 12, 2006; accepted January 17, 2006

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.

Int J Hematol. 2006;83:217-223. doi: 10.1532/IJH97.05187

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 downregulation 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

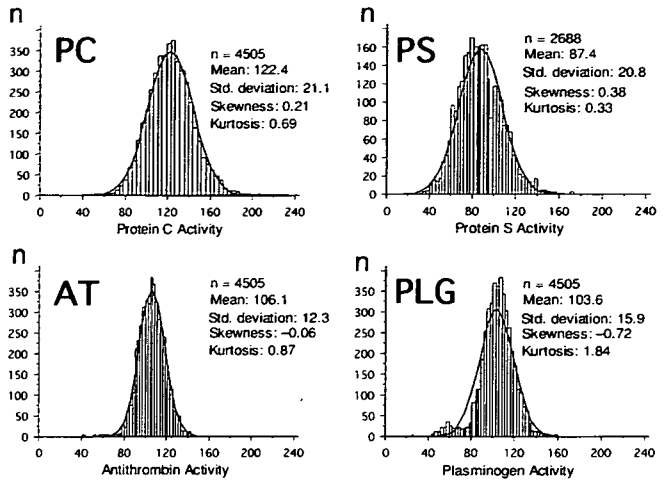

Figure 1. Distribution of plasma activity for protein C (PC), protein S (PS), antithrombin (AT), and plasminogen (PLG) in the Japanese general population. Protein C, antithrombin, and plasminogen activities were measured in 4517 subjects. Protein S activity was measured in 2690 subjects. Mean value,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

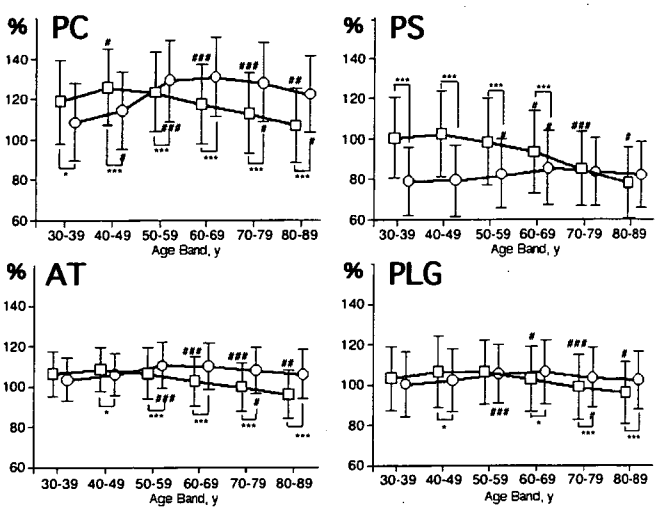

Figure 2. Sex- and age-related differences in plasma activity of protein C (PC), protein S (PS), antithrombin (AT), and plasminogen (PG) in the Japanese general population. Protein C, antithrombin, and plasminogen activity was measured in 2090 men and 2427 women. Protein S activity was measured in 1252 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