J Thromb Thrombolysis (2014) 37:56–65
DOI 10.1007/s11239-013-1005-x

Dysfunction of protein C anticoagulant system, main genetic risk factor for venous thromboembolism in Northeast Asians

Tong Yin · Toshiyuki Miyata

Published online: 15 November 2013

Abstract Venous thromboembolism (VTE) is a life-threatening medical disorder worldwide. A great deal of evidence suggests that prevalence of VTE varies significantly among ethnic populations, with consistently lower incidence found in Asians. While the distribution of genetic risk factors may vary among races, genetic risk factors can play a major role among individuals with different genetic backgrounds. Two clinically evaluated low-frequency genetic mutations that predispose to VTE—the factor V Leiden mutation and prothrombin G20210A mutation—are found predominantly in Caucasians, and virtually never in Asians. The findings of a recent genetic study of VTE in northeast Asians, which greatly advanced our knowledge in this area, indicate that the most frequent genetic risk factors for VTE in northeast Asians can be attributed to a dysfunction of the protein C anticoagulant system. Several low-frequency genetic mutations, *PROS1* p.Lys196Glu in Japanese and *PROC* p.Arg189Trp and p.Lys193del in Chinese, are significantly associated with increased risk for VTE, with odds ratio more than 2 through the reduced protein C anticoagulant activity. Construction of a multifactorial model based on the genetic risk factors in the protein C anticoagulant system could facilitate genetic counseling for VTE risk in these populations. The influence of prevalent genetic mutations on the risk of VTE should be further investigated in Asian countries.

Keywords Asian thrombophilia · Genetic risk factor · Protein C anticoagulant system · Venous thromboembolism

T. Yin
Institute of Geriatric Cardiology, General Hospital of Chinese People's Liberation Army, Beijing 100853, China
e-mail: yintong2000@yahoo.com

T. Miyata (✉)
Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 5658565, Japan
e-mail: miyata@ncvc.go.jp; miyata@ri.ncvc.go.jp

Introduction

Venous thromboembolism (VTE), a multifactorial disorder consisting of deep venous thrombosis (DVT) and pulmonary embolism (PE), represents a major thrombotic medical disorder worldwide. Despite acknowledged problems with different criteria and misclassification in determining VTE, there is strong evidence that the prevalence of VTE varies significantly among different ethnic/racial populations. Among the few studies with sufficiently diverse ethnic population samples to make direct comparisons [1–4] (Table 1), the most notable findings were from epidemiological studies based on ethnically diverse populations in California [1.3.4]. They suggested that the annual incidence of idiopathic DVT in persons over 18 years is higher among African Americans (29 per 100,000 individuals per year) than among Caucasians (23 per 100,000 individuals per year), is significantly lower among Hispanic populations (14 per 100,000 individuals per year), and is strikingly lower among Asian-Pacific Islanders (6 per 100,000 individuals per year) [1]. Population-based epidemiological studies of VTE are relatively rare in Asians. Recently, Sakuma et al. [5] reported the annual estimated incidence of PE and DVT in Japanese to be 6.19 and 11.55 patients per 100,000 individuals per year, respectively. Lee et al. [6] analyzed the incidence of symptomatic VTE in almost the entire population of

Table 1 Prevalence of VTE in diverse ethnic populations

Population origin	Diagnosis	Incidence rate per 100,000 individuals/year					Year	Reference
		African	Caucasian	Hispanics	Asian	Others		
Hospital patients	Idiopathic DVT	29	23	14	6	–	1991–1994	White et al. [1]
General population	PE or DVT	22	21	9	2	15[a]	1978–1985	Klatsky et al. [2]
Hospital patients	Total VTE	141	104	55	21	64	1996	White et al. [3]
Hospital patients	Idiopathic VTE	32	28	15	6	–	1996	White et al. [3]
General population	First-time VTE	141	103	62	29	23	1996	White and Keenan [4]

VTE venous thromboembolism; *DVT* deep vein thrombosis; *PE* pulmonary embolism

[a] In mixed or other ethnic populations

Taiwan. The incidence of PE and DVT among Taiwanese adults was 4.8 and 16.5 per 100,000, respectively, which is lower than among Caucasians and African-Americans, and similar to that of other Asians. Nevertheless, prevalence of PE and DVT in Asia may be increasing with improvements in diagnosis and access to healthcare [7].

VTE is recognized to be a multifactorial, complex disorder, which results from an interaction between environmental, clinical, and genetic risk factors. While racial differences in the incidence of VTE have been well established, interactive risk factors also vary by race [8]. Generally accepted environmental and clinical risk factors for VTE—such as obesity and access to complex surgery and cancer treatments—as well as prevalence of VTE risk factors such as human immunodeficiency virus and the sickle cell trait, are likely to emerge as important mediators of the racial difference in VTE [7]. Data from studies in Asian patients indicate a lower incidence of symptomatic VTE complicating trauma, immobilization, surgery, and/or use of thalidomide [7]. In addition to these well-established risk factors for VTE, it is conceivable that genetic risk factors may vary and play a major role in the different distribution of VTE among people from different genetic backgrounds [8].

The involvement of genetic factors in increased risk for VTE was confirmed in family-based studies in Caucasians, where genetics were thought to account for up to 60 % of risk [9]. Well-established genetic risks for VTE include increased pro-coagulant activities and hereditary deficiencies of natural anticoagulants. Two well-known and clinically evaluated genetic mutations associated with VTE are factor V Leiden mutation (factor V p.Arg506Gln mutation) and prothrombin G20210A mutation, which are found predominantly in populations with European ancestry, and are virtually non-existent in Asians without European admixture [8]. The relatively lower incidence of VTE in Asians compared to Caucasians may partly be due to the lower prevalence of these predisposing genetic factors.

Recently, in northeast Asian populations, where VTE incidence appears low, the most represented genetic risk factors are congenital deficiency of natural anticoagulants, especially genetic deficiency concerning the dysfunction of the protein C anticoagulant system [10]. The purpose of this review is to discuss the prevalence of this deficiency in Asians, and to evaluate the influence of genetic mutations in the protein C anticoagulant system on the risk of VTE in these populations. In this review, the A of the ATG initiator Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1 [11].

Protein C anticoagulant system

Natural anticoagulation in healthy individuals is primarily achieved through the actions of the anticoagulant systems, which include antithrombin, tissue factor pathway inhibitor, protein C, and protein S. Antithrombin plays a major role through the inhibition of thrombin and factor Xa. Tissue factor pathway inhibitor performs its physiological and pathological roles through the inhibition of factor Xa and factor VIIa-tissue factor complex. Unlike these protease inhibitors, the inactive serine-protease zymogen, protein C, must first be activated by thombomodulin (TM)-bound thrombin on the endothelial surface, with the help of the endothelial cell protein C receptor (EPCR); this produces activated protein C (APC) that then proteolytically inactivates factors Va and VIIIa in the presence of protein S. Protein S also stimulates factor Xa inhibition by tissue factor pathway inhibitor, resulting in the down-regulation of the extrinsic coagulation pathway. Thus, the protein C anticoagulant system, consisting of protein C, protein S, TM, and EPCR, regulates the balance between procoagulant and anticoagulant activities. Thrombus formation occurs when this balance is disturbed.

Deficiency of natural anticoagulants and prevalence in Asians

Deficiencies of proteins C and S result in the dysfunction of the protein C anticoagulant system. Hereditary protein C deficiency is usually inherited as an autosomal dominant trait. It is associated with an increased risk of VTE, and is thus considered hereditary thrombophilia. Protein C deficiency is classified into type I (low plasma concentration of both functional and immunological protein C) and type II (low concentration of functional protein C with normal antigen concentration). The inheritance pattern of protein S deficiency is usually autosomal-dominant. Protein S deficiency is classified as type I (quantitative deficiency of both activity and antigen concentration), type II (qualitative deficiency characterized by decreased activity with normal antigen concentration), or type III (normal concentration of total protein S and low concentration of free protein S).

The frequency of deficiencies of protein C, protein S, and antithrombin in VTE patients of Western ethnicity was reported to be 1.4–8.6, 1.4–7.5, and 0.5–4.9 %, respectively [12]. Compared with Caucasians, deficiencies of protein C and protein S in Asians were higher in both the general population and in VTE patients [13–23]. As shown in Table 2, the most prevalent deficiencies in Asian VTE patients were protein S deficiency, followed by protein C deficiency. A report from Hong Kong claimed that as many as 42 % of Chinese VTE patients have reduced activity of the protein C anticoagulant system [13]. In the Taiwanese population, about 50 % of VTE patients showed reduced activity of protein C and protein S [24]. In the Japanese population, the frequency of mutations of the protein C gene was almost three times higher than in Caucasian patients, and protein S deficiency was approximately 5–10 times more prevalent in Japanese VTE patients [20]. These studies suggested that Asian individuals have thrombophilias that differ from those of Caucasians, with a high likelihood of thrombophilia being due to an abnormality of protein C or protein S. They also indicate that there may be an overall higher prevalence of abnormality in protein C or protein S in Asian populations in general, and that a higher occurrence of this class of genetic risk factors may be expected in patients with VTE from the same genetic background [8].

Genetic mutations in the protein C anticoagulant system with VTE in Asians

Recently, several genetic mutations that are associated with a reduction of protein C anticoagulant activity and increased risk for VTE have been confirmed in the protein C anticoagulant system in Japanese and Chinese populations. While differences in VTE by race due to genetic predisposition will probably always be present, understanding the racially specific genetic risk factors for VTE can provide important information about etiological mechanisms, as well as novel therapeutic targets.

PROS1 p.Lys196Glu as a genetic risk of VTE in Japanese

Approximately 8–47 % of Japanese and Chinese individuals who develop VTE have reduced activities of protein S (Table 2). At present, more than 200 mutations have been described in the protein S gene (*PROS1*), and large deletions/duplications can also be identified as causes of protein S deficiency [25, 26]. The most common *PROS1* mutation is a p.Lys196Glu mutation (rs121918474, c.586A>G, protein S Tokushima, p.Lys155Glu in the mature protein numbering), which accounts for 9–30 % of protein S molecule abnormalities in people of Japanese decent [10, 20, 27–29].

An abnormal protein S molecule with the p.Lys196Glu mutation was identified in thrombophilic Japanese patients almost simultaneously by two independent groups in 1993 [30, 31]. It is a missense mutation that causes Lys196 to be replaced by Glu, formerly known as protein S Tokushima. This mutation is present in the second EGF-like domain of the protein S molecule. The allele frequency is approximately 0.9 % in the Japanese population, which means that 1 out of 55 Japanese carries the mutation as a heterozygote [29, 32, 33]. The frequency is much higher, approximately 6–10 % among DVT patients (Table 3) [20, 28, 32]. While homozygotes for this mutation have been identified in VTE patients, with a prevalence of one homozygote out of approximately 85 patients [20, 32], they have thus far not been identified in the general population [29]. The protein S p.Lys196Glu mutation can also be found in VTE patients with congenital protein C deficiency, thereby facilitating the development of VTE [34], and is frequently seen in VTE patients who are pregnant [35]. So far, 3 independent case–control studies, all performed in Japan, have reached the conclusion that the protein S p.Lys196Glu mutation is a risk factor for VTE, with odds ratio between 3.74 and 8.56 [20, 28, 32] (Table 3).

A genotype–phenotype study of the general Japanese population showed that individuals heterozygous for the mutant Glu-allele had a 16 % mean reduction in plasma protein S anticoagulant activity compared to wild-type individuals [27]. A patient with DVT who is a homozygote of protein S p.Lys196Glu mutation showed 35 % protein S anticoagulant activity and 37 % specific anticoagulant activity (activity/amount of protein S) [20]. In vitro studies using the recombinant proteins have shown that mutant protein S with Glu196 had impaired APC cofactor function

Table 2 Prevalence of protein S, protein C, and antithrombin deficiency in Asians

Population	Number of deficiency/total (%)						Reference
	Protein S deficiency		Protein C deficiency		Antithrombin deficiency		
	VTE patients	General population	VTE patients	General population	VTE patients	General population	
Japanese	20/113 (17.70 %)	8/392 (2.02 %)	9/113 (7.96 %)	2/392 (0.51 %)	2/113 (1.7 %)	0/392 (0 %)	Suehisa et al. [16]
Japanese	–	–	7/108 (6.48 %)	6/4,517 (0.13 %)	6/108 (5.56 %)	7/4,517 (0.15 %)	Sakata et al. [19]
Japanese	–	Male: 14/1,252 (1.12 %) Female: 23/1,438 (1.60 %)	–	–	–	–	Sakata et al. [18]
Japanese	40/85 (47.06 %)	1/126 (0.79 %)	27/85 (31.76 %)	1/95 (1.05 %)	6/85 (7.06 %)	0/95 (0 %)	Kinoshita et al. [20]
Chinese	10/52 (19.23 %)	–	9/52 (17.31 %)	–	5/52 (9.62 %)	–	Liu et al. [13]
Chinese	28/85 (32.94 %)	–	16/85 (18.82 %)	–	3/85 (3.53 %)	–	Shen et al. [24]
Chinese	39/116 (33.62 %)	8/125 (6.40 %)	20/116 (17.24 %)	5/125 (40.00 %)	6/116 (5.17 %)	8/125 (6.40 %)	Shen et al. [14]
Chinese	4/50 (8.00 %)	–	2/50 (4.00 %)	–	2/50 (4.00 %)	–	Ho et al. [15]
Chinese	6/56 (10.71 %)	–	6/56 (10.71 %)	–	4/56 (7.14 %)	–	Chen et al. [17]
Chinese	–	2/3,493 (0.06 %)	–	10/3,493 (0.29 %)	–	3/3,493 (0.09 %)	Zhu et al. [22]
Chinese	11/32 (34.40 %) (protein S or protein C deficiency)						Tang et al. [23]
Thai	10/85 (11.76 %)	–	8/85 (9.41 %)	–	4/85 (4.71 %)	–	Angchaisuksiri et al. [21]

– Data unavailable

Table 3 Influence of genetic variants in the protein C anticoagulant system on VTE in Asians

Gene	Nucleotide change	Amino acid change(in mature protein)	rs number	Risk allele	No. of deficiency/total (%)		Odds ratio (95 % CI)	*p* value	Population	Reference
					Cases	Controls				
PROS1	c.586A>G	p.Lys196Glu (p.Lys155Glu)	rs121918474	G	5/85 (5.88)	5/304 (1.64)	3.74 (1.06–13.2)	–	Japanese	Kinoshita et al. [20]
PROS1	c.586A>G	p.Lys196Glu (p.Lys155Glu)	rs121918474	G	15/161 (9.32)	66/3,651 (1.81)	5.58 (3.11–10.01) adjusted: 4.72 (2.39–9.31)	<0.001	Japanese	Kimura et al. [32]
PROS1	c.586A>G	p.Lys196Glu (p.Lys155Glu)	rs121918474	G	6/60 (10.00)	3/234 (1.28)	8.56 (2.07–35.30)	<0.05	Japanese	Ikejiri et al. [28]
PROC	c.565C>T	p.Arg189Trp (p.Arg147Trp)	rs146922325	T	5/116 (4.31)	11/1,292 (0.85)	5.10 (1.7–14.8)	–	Chinese	Tsay et al. [38]
PROC	c.565C>T	p.Arg189Trp (p.Arg147Trp)	rs146922325	T	59/1,003 (5.88)	9/1,031 (0.87)	7.10 (3.50–14.39) adjusted: 7.34 (3.61–14.94)[a] or 7.13 (3.49–14.56)[b]	3.31×10^{-10} adjusted: 3.88×10^{-8} [a] or 6.88×10^{-8} [b]	Chinese	Tang et al. [23], [39]
PROC	c.565C>T	p.Arg189Trp (p.Arg147Trp)	rs146922325	T	68/1,304 (5.21)	12/1,334 (0.90)	6.06 (3.26–11.25)	1.03×10^{-10}	Chinese	Tang et al. [48]
PROC	c.574_576del	p.Lys193del (p.Lys151del)	rs199469469	Del	68/1,003 (6.78)	25/1,031 (2.42)	2.93 (1.84–4.67) adjusted: 2.71 (1.68–4.36)	2.59×10^{-6} adjusted: 4.59×10^{-5}	Chinese	Tang et al. [23]
PROC	c.574_576del	p.Lys193del (p.Lys151del)	rs199469469	Del	85/1,304 (6.52)	32/1,334 (2.40)	2.84 (1.88–4. 29)	2.77×10^{-7}	Chinese	Tang et al. [48]
THBD	c.2729A>C in tight LD with c.1418C>T	In tight LD with p.Ala473Val	rs3176123	C	33/55[c] (60.00)	462/1,032[c] (44.77)	2.76[c] (1.14–6.67)	0.02[c]	Japanese	Sugiyama et al. [47]
THBD	c.−151G>T	–	rs16984852	T	35/1,304 (2.68)	13/1,334 (0.97)	2.80 (1.48–5.32)	1.02×10^{-3}	Chinese	Tang et al. [48]
PROCR	c.4600A>G	p.Ser219Gly	rs867186	G	15/65[d] (23.07)	7/71[d] (9.86)	2.75 (1.04–7.30)	<0.05	Chinese	Chen et al. [52]
PROCR	c.4600A>G	p.Ser219Gly	rs867186	G	41/112 (36.61)	23/112 (20.54)	1.78 (1.11–2.89)	<0.05	Chinese	Yin et al. [53]

CI confidence interval, *LD* linkage disequilibrium, – Data unavailable

[a] Data were analyzed by logistic regression adjusted for age, gender, smoking status, alcohol abuse, malignant tumor, type 2 diabetes, sedentariness/immobilization, and pregnancy/puerperium

[b] Data were calculated by unconditional logistic regression adjusted for age, gender, smoking status, malignant tumor, sedentariness/immobilization, and pregnancy/puerperium

[c] Male patients

[d] Patients with one G allele

[36]. Plasma protein S activities in carriers of the p.Lys196Glu mutation showed reduced activity as described, but antigen levels were within normal limits [27, 30, 31].

The protein S p.Lys196Glu mutation is race-specific; so far this mutation has not been identified in any population other than Japanese. Chinese and Koreans populations, despite being geographically and genetically close to Japanese, did not carry this mutation [37]. Thus, the protein S p.Lys196Glu mutation must be a recent occurrence and fixed within the Japanese population.

PROC p.Arg189Trp and p.Lys193del as genetic risks of VTE in Chinese

At least 161 different protein C gene (*PROC*) mutations have been reported, and most of them are missense mutations. The predominant genetic defects in the *PROC* gene may be different for different races. Recently, the p.Arg189Trp mutation of protein C (rs146922325, c.565C>T, p.Arg147Trp in the mature protein numbering) was reported by two independent studies to be not only the most frequent variant for protein C deficiency but also a significant risk factor for VTE in Chinese populations [38, 39]. This missense mutation was initially described in an American patient with symptomatic protein C deficiency [40], and was later reported in an asymptomatic individual [41]. Although a rare mutation in Western populations, the p.Arg189Trp mutation was present in approximately 0.9 % of the general Chinese population (Table 3) [38, 39]. The heterozygous state of the p.Arg189Trp mutation is associated with decreased plasma functional activity and a relatively normal protein C antigen level, indicating type II protein C deficiency. This mutation was identified in almost half of the probands with hereditary protein C deficiency [38, 39]. First-degree relatives bearing this variant had an 8.8-fold increased risk of VTE [39]. Two independent population-based case–control studies showed the odds ratio of VTE in carriers of the variant ranged from 5 to 7 (Table 3) [38, 39]. The p.Arg189Trp mutation is located at the C-terminal region of the light chain adjacent to the EGF-2 like domain, and may impair the interaction of protein C with other molecules suggesting that Arg189 may constitute an exosite for the binding of factor Va and/or the thrombin–thrombomodulin complex. Further functional studies are needed to elucidate the deleterious effect of this mutation on the activation of protein C and the inactivation of factor Va by APC. Data on the prevalence of this mutation and the thrombotic risk associated with it in other populations (especially other Asian populations) are still quite limited, and should be further evaluated.

Recently, using coagulation screening tests, resequencing, and a case–control study, Tang et al. revealed that the *PROC* p.Lys193del mutation (rs199469469, c.574_576del, p.Lys151del in the mature protein numbering) was associated with both decreased protein C anticoagulant activity and an increased risk of VTE in Chinese, with an odds ratio of 2.7 (Table 3) [23]. The nomenclature of one amino acid deletion in this case is somewhat complicated, as positions 192 and 193 of protein C are both Lys and one of the Lys residues is deleted in this case. We call the mutation the "p.Lys193del" according to the recommendation of the Human Genome Variation Society [11]. This variant was first described in three Japanese patients who suffered from protein C deficiency [42]. In other studies on protein C and protein S deficiencies in Japanese individuals, this mutation was identified in 2 of 85 VTE patients, as well as in 1 of 30 healthy individuals in one study [20], and in 4 of 173 VTE patients in another [34]. Despite being identified as a rare genetic mutation in Japanese, the contribution of the variant to the risk for VTE was not further evaluated in the general Japanese population. Another recent study found that the prevalence of p.Lys193del mutation was 2.36 % in the general Chinese population [23]. It was identified in 68 of 1,003 VTE patients (6.78 %) and in 25 of 1,031 healthy individuals (2.42 %), therefore, it conferred an increased risk of VTE with an adjusted odds ratio of 2.7 (Table 3) [23]. Patients with the p.Lys193del mutation showed lower anticoagulant activity of protein C, but relatively normal amidolytic activity compared to the wild-type carriers [23, 34, 42]. The anticoagulant activity of the recombinant mutant protein C showed about 40 % of the wild-type, consistent with the value of plasma from the homozygous patient [23]. Although this mutation has been reported previously in Japanese populations, further studies are needed to evaluate its prevalence in other Asians, and to determine whether this polymorphism is a risk factor for VTE in other Asian populations.

Some other *PROC* mutations were also reported in VTE patients from Asia [39, 40, 43–45]. Both protein C p.Arg211Trp and p.Met406Ile (p.Arg169Trp and p.Met364Ile in the mature protein numbering), which are related to type I protein C deficiency, were first reported in Japanese patients with VTE [43, 44]. Protein C p.Arg211Trp is a recurrent mutation occurring at a CpG mutation hotspot at the thrombin cleavage site in the heavy chain; it has also been described in Caucasian patients with VTE. It was reported to account for about 10 % of *PROC* mutations in Japanese [45]. In contrast, p.Met406Ile, which occurs at a non-CpG site of the serine protease domain, has been described exclusively in Japan, accounting for ~8 % of *PROC* mutations in Japanese VTE patients [45]. In resequencing the *PROC* gene in probands of protein C deficiency, 8 novel coding sequence mutations contributed to 7 amino acid exchanges; 3 evidently detrimental novel null mutations were also supposed to contribute to the development of VTE in Chinese [39].

THBD mutations as VTE risk in Asians

Thrombomodulin (TM encoded by *THBD*), another critical component of the protein C anticoagulant system, is a transmembrane glycoprotein of 557 amino acids, and is expressed mainly on the endothelial cells. TM binds thrombin and alters its substrate specificity. The resulting TM–thrombin complex efficiently catalyzes protein C activation. The intron-less human *THBD* gene is 3.6 kb in length. Based on the important anticoagulant role of TM, mutations within *THBD* could predispose individuals to VTE. In addition, *THBD* mutations may affect the plasma-soluble TM level. Several studies have focused on the influence of genetic polymorphisms in *THBD* on soluble TM level and VTE. One study conducted in the USA found mutations—including c.127G>A (p.Ala43Thr), c.1418C>T (p.Ala473Val), c.1752C deletion, and c.3645A>G—were not associated with VTE [46]. An association study of the Japanese population that included 2,247 individuals showed that c.2729A>C in tight linkage disequilibrium with c.1418C>T (p.Ala473Val) was associated with the soluble TM level [47]. This mutation also showed a marginal association with VTE, but only in males (Table 3).

A recent large study of the Chinese population showed an association of the soluble TM levels with c.−151G>T in *THBD* (Table 3) [48]. Furthermore, this genetic mutation increased risk of VTE. The study enrolled 1,304 individuals with VTE and 1,334 age- and sex-matched controls. By resequencing and genotyping of the *THBD* gene, the study showed that c.−151G>T in *THBD* could cause a predisposition to VTE, with a 2.8-fold increased risk of developing VTE in the population and a 3.42-fold increased risk of VTE in the family [48]. The prevalence of this variant in the Chinese population was 0.97 %, indicating an allele frequency of 0.49 %. Compared with the wild-type allele, the c.−151G>T mutation significantly reduced the reporter gene-expression level in cultured cells [48].

In addition, rare nonsynonymous mutations, p.Ser190Trp, p.Ser212Ter, p.Leu220Ter, and p.Asp126Tyr in *THBD* were also identified in 108 thrombophilic individuals with VTE [48]. The prevalence and relative risk of VTE with these mutations in other populations, especially in Asians, will require further evaluation.

The extensive resequencing studies on *THBD* in VTE patients revealed yet another aspect of the *THBD* mutations, that is, a possible link between the nonsynonymous mutations and atypical hemolytic uremic syndrome (aHUS), a type of microangiopathy characterized by uncontrolled complement activation. One of the causative genes for aHUS is *THBD* [49]. In vitro, TM binds to C3b and complement factor H and negatively regulates the complement by accelerating complement factor I-mediated inactivation of C3b. The TM mutations were less effective than wild-type TM in enhancing factor I-mediated inactivation of C3b. Thus, some missense mutations of TM are characterized as causative for the development of aHUS [49]. A missense mutation, p.Asp486Tyr, in the Ser/Thr rich domain of TM, which has been identified in both VTE patients and controls [46–48], was characterized as a causative mutation for aHUS. Missense mutations in the lectin-like domain of TM are also reportedly causative for aHUS. Thus, nonsynonymous mutations in *THBD* would affect not only VTE but also aHUS to a certain degree.

PROCR mutations as VTE risk in Asians

On the pathway of the protein C anticoagulant system, protein C is activated on the endothelial surface by the membrane-bound TM–thrombin complex. Protein C activation is enhanced approximately 20-fold when protein C binds to the endothelial protein C receptor (EPCR) encoded by *PROCR*. EPCR also serves as a cellular binding site for factor VII and factor VIIa. A soluble form of this receptor (sEPCR) in plasma inhibits both APC activity and protein C activation by competing for protein C with membrane-bound EPCR. These findings suggest an important role for EPCR in VTE.

Several studies have reported that the *PROCR* p.Ser219Gly mutation (rs867186, c.655A>G) present within the membrane-spanning region reduced plasma sEPCR levels to 56–87 % [50]. Significantly higher levels of factor VII, factor VIIa, and downstream markers of activated coagulation in the extrinsic pathway (factor IX activation peptide, factor X activation peptide), and prothrombin F1 + 2 were also identified in Gly carriers, compared to Ser/Ser [51]. Evidence for the association between the p.Ser219Gly mutation and VTE is conflicting in ethnically diverse populations. A recent meta-analysis in 4,821 VTE patients and 6,070 controls found a significant association of this mutation with VTE [50]. Under an additive genetic model, the odds of VTE increased by a factor of 1.22 for every additional copy of the G allele in all ethnic populations, suggesting a moderate effect for VTE. The reported frequency of the G allele in northeast Asians is approximately 10 % [52, 53]. Thus far, only two independent, small-scale studies of Chinese populations have reported a significant association between the p.Ser219Gly mutation and VTE in Asian populations (Table 3) [52, 53]. Further studies restricted to idiopathic VTE patients in Asian might facilitate the positive association of this variant.

Perspectives

The genetic mutations in the protein C anticoagulant system (*PROS1* p.Lys196Glu, *PROC* p.Arg189Trp, *PROC*

p.Lys193del, and *THBD* c.−151G>T) associated with risk of VTE in Asians are all classified into low-frequency variations with allele frequencies of less than 5 %. Three genetic mutations in the protein C anticoagulant system (*PROC* p.Arg189Trp, *PROC* p.Lys193del, and *THBD* c.−151G>T) were detected concurrently in the Chinese population, with a respective frequency of 0.90, 2.40, and 0.97 %, and a respective odds ratio for VTE of 6.06, 2.84, and 2.80. Their estimated population-attributable risks were therefore calculated to be 4.67, 4.14, and 1.48 %, respectively [48]. Taken together, about 10 % of VTE events in the general Chinese population could be explained by these mild to moderate thrombophilic risk factors. Hence, as we have described [29], these low-frequency genetic variations could play an important role in the development of VTE. The risk loci may act in concert with each mutation adding or detracting a small amount from the phenotype; the environment also interacts with the genotype to produce the final phenotype [8].

Recent genome-wide association studies have found additional genetic polymorphisms that are potentially related to VTE risk, but most have been detected predominately in European-ancestry populations [54, 55]. Genome-wide association studies do serve an important role in identifying new loci of interest, as well as confirming previously suggested loci for VTE. However, their main potential is for identifying common mutations (>5 %) with relatively lower risk (odds ratio <1.5). The candidate gene resequencing in the protein C anticoagulant system or the exome sequencing would facilitate the discovering of low-frequency variations with high risk for VTE in Asians. An accumulating body of evidence strongly suggests that genetic studies should be carried out in ethnically diverse populations, and that studies of common variations, as well as low-frequency variations, are warranted [29].

As VTE is a complex disease with genetic factors accounting for part of the risk, a multifactorial non-Mendelian inheritance model that includes the influence of genetic and environmental factors should be proposed for genetic counseling of VTE risk. Recently, a multiple single-nucleotide polymorphism test based on 31 VTE-associated polymorphisms or the 5 most strongly associated polymorphisms was found to improve risk prediction of first venous thrombosis in Caucasians [56]. Future studies should consider the construction of a multifactorial model based on the genetic risk factors in the protein C anticoagulant system, which is specific for Asian populations.

In summary, the genetic mutations leading to dysfunction of the protein C anticoagulant system could be a major risk factor for VTE in northeast Asian populations, especially in Japanese and Chinese. Conditions where the procoagulant activity surpasses the anticoagulant activity, including the protein C anticoagulant system, could trigger the development of thrombosis in individuals with risk genetic variants. Genetic analysis for VTE is highly restricted in Japanese and Chinese populations, and other Asian populations are not yet well studied. Even in geographically close populations, such as Japanese and Chinese, low-frequency mutations are not evenly distributed. The *PROS1* p.Lys196Glu mutation, for example, is exclusively identified in Japanese populations. Whether dysfunction of the protein C anticoagulant system occurs in other Asian countries is an important unresolved issue of the thrombophilia study among Asians, and an international survey is warranted to disclose it.

Acknowledgments This work was supported in part by grants-in-aid from the Ministry of Health, Labour, and Welfare of Japan; the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Japan Society for the Promotion of Science, and by grants from the Clinical Research Supportive Fund, General Hospital of Chinese People's Liberation Army (No. 2012FC-TSYS-3042).

Conflict of interest The authors have no relevant disclosures.

References

1. White RH, Zhou H, Romano PS (1998) Incidence of idiopathic deep venous thrombosis and secondary thromboembolism among ethnic groups in California. Ann Intern Med 128(9):737–740
2. Klatsky AL, Armstrong MA, Poggi J (2000) Risk of pulmonary embolism and/or deep venous thrombosis in Asian-Americans. Am J Cardiol 85(11):1334–1337
3. White RH, Zhou H, Murin S, Harvey D (2005) Effect of ethnicity and gender on the incidence of venous thromboembolism in a diverse population in California in 1996. Thromb Haemost 93(2):298–305
4. White RH, Keenan CR (2009) Effects of race and ethnicity on the incidence of venous thromboembolism. Thromb Res 123(Suppl 4):S11–S17
5. Sakuma M, Nakamura M, Yamada N, Ota S, Shirato K, Nakano T, Ito M, Kobayashi T (2009) Venous thromboembolism: deep vein thrombosis with pulmonary embolism, deep vein thrombosis alone, and pulmonary embolism alone. Circ J 73(2):305–309
6. Lee CH, Cheng CL, Lin LJ, Tsai LM, Yang YH (2011) Epidemiology and predictors of short-term mortality in symptomatic venous thromboembolism. Circ J 75(8):1998–2004
7. Zakai NA, McClure LA (2011) Racial differences in venous thromboembolism. J Thromb Haemost 9(10):1877–1882
8. Margaglione M, Grandone E (2011) Population genetics of venous thromboembolism. A narrative review. Thromb Haemost 105(2):221–231
9. Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, Coll I, Felices R, Stone W, Fontcuberta J, Blangero J (2000) Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic analysis of idiopathic thrombophilia. Am J Hum Genet 67(6):1452–1459
10. Hamasaki N, Kuma H, Tsuda H (2013) Activated protein C anticoagulant system dysfunction and thrombophilia in Asia. Ann Lab Med 33(1):8–13
11. den Dunnen JT, Antonarakis SE (2000) Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mut 15(1):7–12

12. De Stefano V, Finazzi G, Mannucci PM (1996) Inherited thrombophilia: pathogenesis, clinical syndromes, and management. Blood 87(9):3531–3544
13. Liu HW, Kwong YL, Bourke C, Lam CK, Lie AK, Wei D, Chan LC (1994) High incidence of thrombophilia detected in Chinese patients with venous thrombosis. Thromb Haemost 71(4):416–419
14. Shen MC, Lin JS, Tsay W (2000) Protein C and protein S deficiencies are the most important risk factors associated with thrombosis in Chinese venous thrombophilic patients in Taiwan. Thromb Res 99(5):447–452
15. Ho CH, Chau WK, Hsu HC, Gau JP, Yu TJ (2000) Causes of venous thrombosis in fifty Chinese patients. Am J Hematol 63(2):74–78
16. Suehisa E, Nomura T, Kawasaki T, Kanakura Y (2001) Frequency of natural coagulation inhibitor (antithrombin III, protein C and protein S) deficiencies in Japanese patients with spontaneous deep vein thrombosis. Blood Coagul Fibrinolysis 12(2):95–99
17. Chen TY, Su WC, Tsao CJ (2003) Incidence of thrombophilia detected in southern Taiwanese patients with venous thrombosis. Ann Hematol 82(2):114–117
18. Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T (2004) Prevalence of protein S deficiency in the Japanese general population: the Suita study. J Thromb Haemost 2(6):1012–1013
19. Sakata T, Okamoto A, Mannami T, Matsuo H, Miyata T (2004) Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese. J Thromb Haemost 2(3):528–530
20. Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, Tsuda H, Kang D, Hamasaki N (2005) Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. Clin Biochem 38(10):908–915
21. Angchaisuksiri P, Atichartakarn V, Aryurachai K, Archararit N, Rachakom B, Atamasirikul K, Tiraganjana A (2007) Risk factors of venous thromboembolism in Thai patients. Int J Hematol 86(5):397–402
22. Zhu T, Ding Q, Bai X, Wang X, Kaguelidou F, Alberti C, Wei X, Hua B, Yang R, Wang X, Wang Z, Ruan C, Schlegel N, Zhao Y (2011) Normal ranges and genetic variants of antithrombin, protein C and protein S in the general Chinese population. Results of the chinese hemostasis investigation on natural anticoagulants study I group. Haematologica 96(7):1033–1040
23. Tang L, Lu X, Yu JM, Wang QY, Yang R, Guo T, Mei H, Hu Y (2012) *PROC* c.574_576del polymorphism: a common genetic risk factor for venous thrombosis in the Chinese population. J Thromb Haemost 10(10):2019–2026
24. Shen MC, Lin JS, Tsay W (1997) High prevalence of antithrombin III, protein C and protein S deficiency, but no factor V Leiden mutation in venous thrombophilic Chinese patients in Taiwan. Thromb Res 87(4):377–385
25. Gandrille S, Borgel D, Sala N, Espinosa-Parrilla Y, Simmonds R, Rezende S, Lind B, Mannhalter C, Pabinger I, Reitsma PH, Formstone C, Cooper DN, Saito H, Suzuki K, Bernardi F, Aiach M (2000) Protein S deficiency: a database of mutations—summary of the first update. Thromb Haemost 84(5):918
26. Yin T, Takeshita S, Sato Y, Sakata T, Shin Y, Honda S, Kawasaki T, Tsuji H, Kojima T, Madoiwa S, Sakata Y, Murata M, Ikeda Y, Miyata T (2007) A large deletion of the *PROS1* gene in a deep vein thrombosis patient with protein S deficiency. Thromb Haemost 98(4):783–789
27. Kimura R, Sakata T, Kokubo Y, Okamoto A, Okayama A, Tomoike H, Miyata T (2006) Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers. J Thromb Haemost 4(9):2010–2013
28. Ikejiri M, Wada H, Sakamoto Y, Ito N, Nishioka J, Nakatani K, Tsuji A, Yamada N, Nakamura M, Ito M, Nobori T (2010) The association of protein S Tokushima-K196E with a risk of deep vein thrombosis. Int J Hematol 92(2):302–305
29. Miyata T, Hamasaki N, Wada H, Kojima T (2012) More on: racial differences in venous thromboembolism. J Thromb Haemost 10(2):319–320
30. Yamazaki T, Sugiura I, Matsushita T, Kojima T, Kagami K, Takamatsu J, Saito H (1993) A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. Thromb Res 70(5):395–403
31. Shigekiyo T, Uno Y, Kawauchi S, Saito S, Hondo H, Nishioka J, Hayashi T, Suzuki K (1993) Protein S Tokushima: an abnormal protein S found in a Japanese family with thrombosis. Thromb Haemost 70(2):244–246
32. Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, Kojima T, Murata M, Nishigami K, Chiku M, Hayashi T, Kokubo Y, Okayama A, Tomoike H, Ikeda Y, Miyata T (2006) Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. Blood 107(4):1737–1738
33. Miyata T, Kimura R, Kokubo Y, Sakata T (2006) Genetic risk factors for deep vein thrombosis among Japanese: importance of protein S K196E mutation. Int J Hematol 83(3):217–223
34. Miyata T, Sato Y, Ishikawa J, Okada H, Takeshita S, Sakata T, Kokame K, Kimura R, Honda S, Kawasaki T, Suehisa E, Tsuji H, Madoiwa S, Sakata Y, Kojima T, Murata M, Ikeda Y (2009) Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis. Thromb Res 124(1):14–18
35. Neki R, Fujita T, Kokame K, Nakanishi I, Waguri M, Imayoshi Y, Suehara N, Ikeda T, Miyata T (2011) Genetic analysis of patients with deep vein thrombosis during pregnancy and postpartum. Int J Hematol 94(2):150–155
36. Hayashi T, Nishioka J, Suzuki K (1995) Molecular mechanism of the dysfunction of protein S(Tokushima) (Lys155→Glu) for the regulation of the blood coagulation system. Biochim Biophys Acta 1272(3):159–167
37. Liu W, Yin T, Okuda H, Harada KH, Li Y, Xu B, Yang J, Wang H, Fan X, Koizumi A, Miyata T (2013) Protein S K196E mutation, a genetic risk factor for venous thromboembolism, is limited to Japanese. Thromb Res 132(2):314–315
38. Tsay W, Shen MC (2004) R147W mutation of PROC gene is common in venous thrombotic patients in Taiwanese Chinese. Am J Hematol 76(1):8–13
39. Tang L, Guo T, Yang R, Mei H, Wang H, Lu X, Yu J, Wang Q, Hu Y (2012) Genetic background analysis of protein C deficiency demonstrates a recurrent mutation associated with venous thrombosis in Chinese population. PLoS ONE 7(4):e35773
40. Tsay W, Greengard JS, Montgomery RR, McPherson RA, Fucci JC, Koerper MA, Coughlin J, Griffin JH (1993) Genetic mutations in ten unrelated American patients with symptomatic type 1 protein C deficiency. Blood Coagul Fibrinolysis 4(5):791–796
41. Tait RC, Walker ID, Reitsma PH, Islam SI, McCall F, Poort SR, Conkie JA, Bertina RM (1995) Prevalence of protein C deficiency in the healthy population. Thromb Haemost 73(1):87–93
42. Miyata T, Sakata T, Yasumuro Y, Okamura T, Katsumi A, Saito H, Abe T, Shirahata A, Sakai M, Kato H (1998) Genetic analysis of protein C deficiency in nineteen Japanese families: five recurrent defects can explain half of the deficiencies. Thromb Res 92(4):181–187
43. Matsuda M, Sugo T, Sakata Y, Murayama H, Mimuro J, Tanabe S, Yoshitake S (1988) A thrombotic state due to an abnormal protein C. N Engl J Med 319(19):1265–1268
44. Miyata T, Zheng YZ, Sakata T, Tsushima N, Kato H (1994) Three missense mutations in the protein C heavy chain causing type I and type II protein C deficiency. Thromb Haemost 71(1):32–37

45. Sakata T, Kario K, Katayama Y, Matsuyama T, Kato H, Miyata T (2000) Studies on congenital protein C deficiency in Japanese: prevalence, genetic analysis, and relevance to the onset of arterial occlusive diseases. Semin Thromb Haemost 26(1):11–16
46. Heit JA, Petterson TM, Owen WG, Burke JP, DE Andrade M, Melton LJ 3rd (2005) *Thrombomodulin* gene polymorphisms or haplotypes as potential risk factors for venous thromboembolism: a population-based case–control study. J Thromb Haemost 3(4):710–717
47. Sugiyama S, Hirota H, Kimura R, Kokubo Y, Kawasaki T, Suehisa E, Okayama A, Tomoike H, Hayashi T, Nishigami K, Kawase I, Miyata T (2007) Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population. Thromb Res 119(1):35–43
48. Tang L, Wang HF, Lu X, Jian XR, Jin B, Zheng H, Li YQ, Wang QY, Wu TC, Guo H, Liu H, Guo T, Yu JM, Yang R, Yang Y, Hu Y (2013) Common genetic risk factors for venous thrombosis in the Chinese population. Am J Hum Genet 92(2):177–187
49. Delvaeye M, Noris M, De Vriese A, Esmon CT, Esmon NL, Ferrell G, Del-Favero J, Plaisance S, Claes B, Lambrechts D, Zoja C, Remuzzi G, Conway EM (2009) Thrombomodulin mutations in atypical hemolytic-uremic syndrome. New Engl J Med 361(4):345–357
50. Dennis J, Johnson CY, Adediran AS, de Andrade M, Heit JA, Morange PE, Tregouet DA, Gagnon F (2012) The endothelial protein C receptor (*PROCR*) Ser219Gly variant and risk of common thrombotic disorders: a HuGE review and meta-analysis of evidence from observational studies. Blood 119(10):2392–2400
51. Ireland HA, Cooper JA, Drenos F, Acharya J, Mitchell JP, Bauer KA, Morrissey JH, Esnouf MP, Humphries SE (2009) FVII, FVIIa, and downstream markers of extrinsic pathway activation differ by EPCR Ser219Gly variant in healthy men. Arterioscler Thromb Vasc Biol 29(11):1968–1974
52. Chen XD, Tian L, Li M, Jin W, Zhang HK, Zheng CF (2011) Relationship between endothelial cell protein C receptor gene 6936A/G polymorphisms and deep venous thrombosis. Chin Med J (Engl) 124(1):72–75
53. Yin G, Jin X, Ming H, Zheng X, Zhang D (2012) Endothelial cell protein C receptor gene 6936A/G polymorphism is associated with venous thromboembolism. Exp Ther Med 3(6):989–992
54. Bezemer ID, Bare LA, Doggen CJ, Arellano AR, Tong C, Rowland CM, Catanese J, Young BA, Reitsma PH, Devlin JJ, Rosendaal FR (2008) Gene variants associated with deep vein thrombosis. JAMA 299(11):1306–1314
55. Tregouet DA, Heath S, Saut N, Biron-Andreani C, Schved JF, Pernod G, Galan P, Drouet L, Zelenika D, Juhan-Vague I, Alessi MC, Tiret L, Lathrop M, Emmerich J, Morange PE (2009) Common susceptibility alleles are unlikely to contribute as strongly as the FV and ABO loci to VTE risk: results from a GWAS approach. Blood 113(21):5298–5303
56. de Haan HG, Bezemer ID, Doggen CJ, Le Cessie S, Reitsma PH, Arellano AR, Tong CH, Devlin JJ, Bare LA, Rosendaal FR, Vossen CY (2012) Multiple SNP testing improves risk prediction of first venous thrombosis. Blood 120(3):656–663

Molecular Genetics & Genomic Medicine

Open Access

ORIGINAL ARTICLE

Candidate gene analysis using genomic quantitative PCR: identification of *ADAMTS13* large deletions in two patients with Upshaw-Schulman syndrome

Yuka Eura[1], Koichi Kokame[1], Toshiro Takafuta[2], Ryojiro Tanaka[3], Hikaru Kobayashi[4], Fumihiro Ishida[5], Shuichi Hisanaga[6], Masanori Matsumoto[7], Yoshihiro Fujimura[7] & Toshiyuki Miyata[1]

[1]Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan
[2]Department of Hematology and Clinical Immunology, Nishi-Kobe Medical Center, Kobe, Hyogo, Japan
[3]Department of Nephrology, Hyogo Prefectural Kobe Children's Hospital, Kobe, Hyogo, Japan
[4]Department of Hematology, Nagano Red Cross Hospital, Nagano, Japan
[5]Department of Biomedical Laboratory Sciences, Shinshu University School of Medicine, Matsumoto, Nagano, Japan
[6]Department of Nephrology, Koga General Hospital, Miyazaki, Japan
[7]Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara, Japan

Keywords
ADAMTS13, genetic analysis, hereditary disease, mutation, quantitative PCR, thrombotic thrombocytopenic purpura, Upshaw-Schulman syndrome

Correspondence
Koichi Kokame, Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Suita, Osaka 565-8565, Japan.
Tel: +81 6 6833 5012; Fax: +81 6 6835 1176; E-mail: kame@ncvc.go.jp

Funding Information
Y. E. was a research resident supported by the Association for Preventive Medicine of Japan at the National Cerebral and Cardiovascular Center. This work was supported in part by grants from the Ministry of Health, Labour, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; from Japan Society for the Promotion of Science; and from the Takeda Science Foundation.

Received: 1 November 2013; Revised: 18 December 2013; Accepted: 19 December 2013

doi: 10.1002/mgg3.64

Abstract

Direct sequencing is a popular method to discover mutations in candidate genes responsible for hereditary diseases. A certain type of mutation, however, can be missed by the method. Here, we report a comprehensive genomic quantitative polymerase chain reaction (qPCR) to complement the weakness of direct sequencing. Upshaw-Schulman syndrome (USS) is a recessively inherited disease associated with severe deficiency of plasma ADAMTS13 activity. We previously analyzed *ADAMTS13* in 47 USS patients using direct sequencing, and 44 of them had either homozygous or compound heterozygous mutations. Then, we sought to reveal more extensive defects of *ADAMTS13* in the remaining three patients. We quantified copy numbers of each *ADAMTS13* exon in the patients by using genomic qPCR. Each primer pair was designed to contain at least one of the two primers used in direct sequencing, to avoid missing any exonic deletions. The qPCR demonstrated heterozygous loss of exons 7 and 8 in one patient and exon 27 in the other, and further analysis revealed c.746_987+373del1782 and c.3751_3892+587del729, respectively. Genomic qPCR provides an effective method for identifying extensive defects of the target genes.

Target exon resequencing using direct sequencing is a popular method to discover causative mutations in the candidate genes responsible for hereditary diseases. Homozygous or compound heterozygous mutations are often identified in the corresponding genes of the patients with autosomal-recessive diseases. In some cases, however, only one or no causative mutation is identified in the responsible gene: (an)other mutation(s) may be

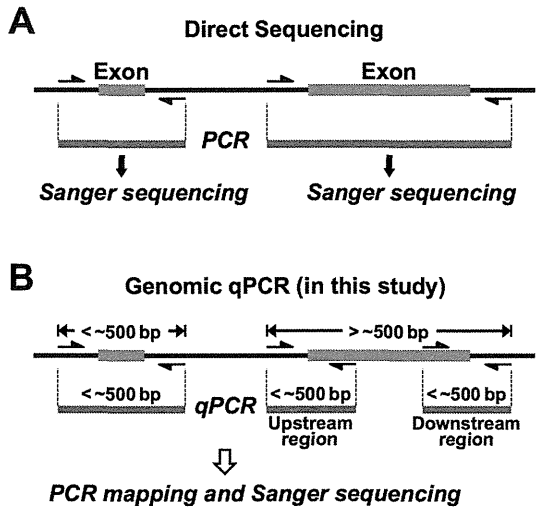


Figure 1. Principles of direct sequencing and genomic qPCR for genetic analysis. (A) In direct sequencing, target regions are amplified by PCR using primer pairs (arrows) usually designed from the intronic sequences flanking each exon, and the PCR products are directly sequenced by the Sanger method. (B) In genomic qPCR, copy numbers of target regions are quantified by real-time PCR. Each primer pair contains at least one of the two primers used in direct sequencing: common primer pairs are used for the regions smaller than ~500 bp, and, for accurate qPCR, one common and one specific primer are used for the regions larger than ~500 bp. If abnormal copy numbers are detected, PCR mapping and sequencing are performed to determine the precise sites of defects.

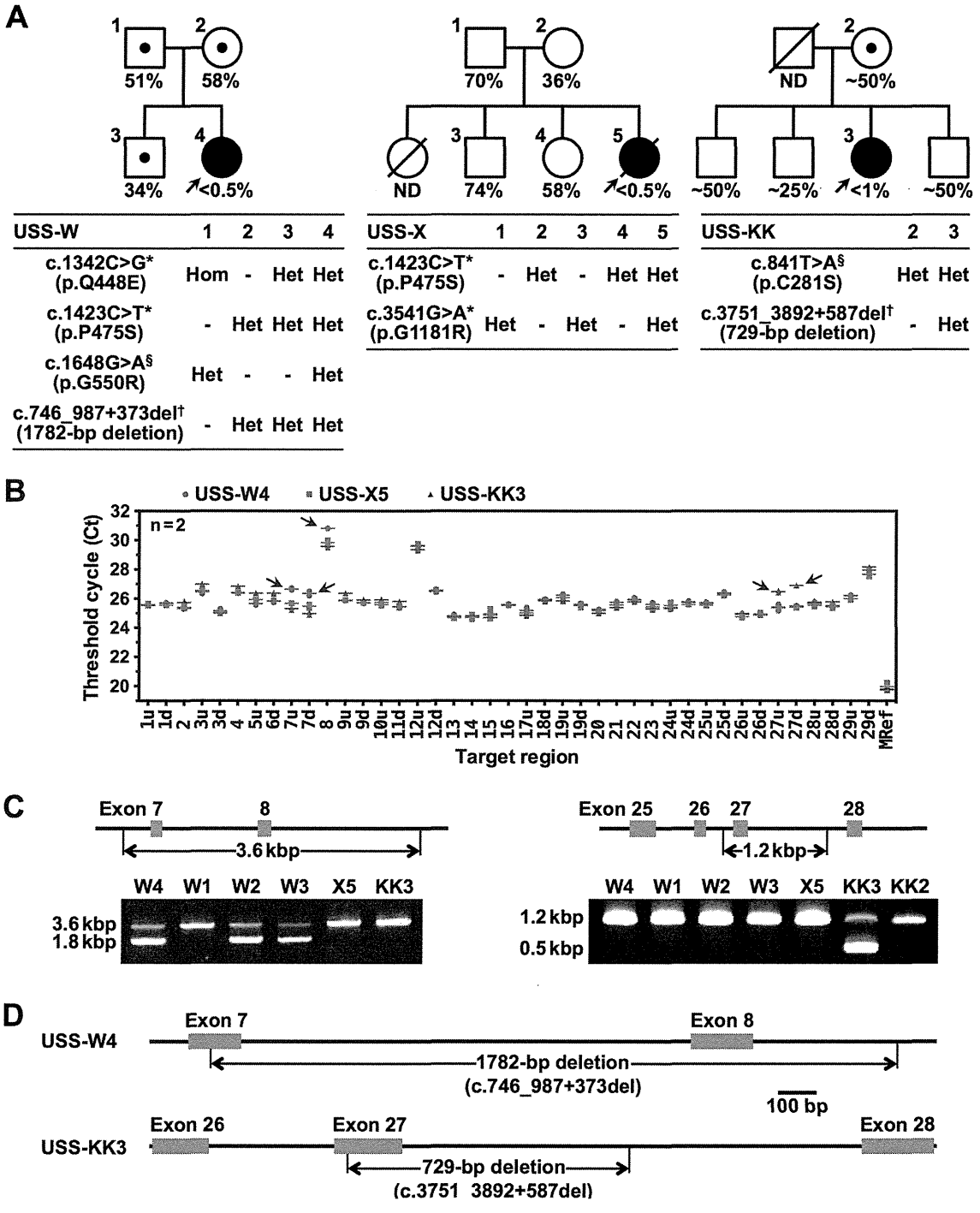
missed by the method. Although next-generation sequencing may be useful in such cases, it needs special equipments and is still expensive. In this study, we report a comprehensive genomic quantitative PCR (qPCR), which will be a powerful tool in combination with direct sequencing.

Upshaw-Schulman syndrome (USS), also called hereditary thrombotic thrombocytopenic purpura (TTP), is an autosomal-recessive trait associated with severely deficient plasma ADAMTS13 activity. Homozygous or compound heterozygous mutations in the *ADAMTS13* gene (OMIM 604134) are identified in most patients with USS (Levy et al. 2001; Kokame et al. 2002; Kokame and Miyata 2004; Matsumoto et al. 2004; Lotta et al. 2010; Fujimura et al. 2011; Hing et al. 2013). So far, more than 130 causative mutations have been identified by direct sequencing. Using that method, we previously analyzed *ADAMTS13* in 47 Japanese USS patients from 41 unrelated families (Fujimura et al. 2011). Of those, 44 patients from 38 families had either homozygous or compound heterozygous mutations in *ADAMTS13*. In the remaining three patients, however, only single missense mutations (two patients) or no mutation (one patient) was detected. In this study, we sought to reveal more extensive defects of *ADAMTS13* in these three patients by using genomic qPCR.

In general, PCR primer pairs for direct sequencing are designed to hybridize within the intronic sequences flanking each exon (Fig. 1A). Mutations such as substitutions, insertions, and deletions occurring in exons and exon–intron boundaries are identified by Sanger sequencing following genomic PCR, regardless of their heterozygosity or homozygosity (Fig. S1A). Direct sequencing, however, misses heterozygous mutations on the allele that contains no or mismatched primer target sequences: not only whole or partial deletion but also point mutations including single-nucleotide polymorphisms of primer target sequences can hamper PCR-amplification of the mutant allele, which may contain other critical mutations in the exon or exon–intron boundary (Fig. S1B). In these cases, only the target region of the other (normal) allele is PCR-amplified and sequenced, and the results are interpreted as if the regions of both alleles are normal.

Copy number analysis may overcome the limitations of direct sequencing. Multiplex ligation-dependent probe amplification (MLPA) analysis (Schouten et al. 2002) is often used for this purpose. Although MLPA is suitable for detection of genetic defects including exon deletions and duplications, it may still miss mutations that occur outside the probe target sequences. Therefore, to comple-

Figure 2. Genetic analysis of three USS families. (A) Pedigrees and genotypes of the USS patient families. Circles with arrows indicate the probands, USS-W4, -X5, and -KK3. Clinical data of the patients and the basis of diagnosis were described previously (Fujimura et al. 2011); the description of USS-KK3 being the second of three children needs to be corrected. Plasma ADAMTS13 activities were measured by us (USS-W and -X) or by Dr. Miha Furlan at University of Bern in 1999 (USS-KK), and are shown as a percentage of the normal control. ND, not determined. No subjects had ADAMTS13 inhibitors. Squares and circles with numbers indicate the subjects for genetic analysis. Each mutation was assigned a name for cDNA according to the nomenclature recommendations of the HGVS (http://www.hgvs.org/mutnomen/) based on the reference sequences AB069698.2 (cDNA) and NC_000009.11 (genomic). *,§Missense substitutions identified by direct sequencing. †Deletions identified by genomic qPCR in this study. *Pathologically unrelated missense polymorphisms. (B) Identification of exon deletions in *ADAMTS13*. Ct values of genomic qPCR are plotted by dots with lines at the mean (n = 2) for each target region. The letters u and d following the exon numbers indicate *upstream* and *downstream* region of each exon, respectively. Red circles, USS-W4; green squares, USS-X5; blue triangles, USS-KK3. Arrows indicate the dots with Ct values higher than those of the other two patients. (C) *Left*: PCR-amplification of the 3.6-kbp band from the normal *ADAMTS13* allele produced a 1.8-kbp band from USS-W4, her mother (W2) and her brother (W3), but not from her father (W1). *Right*: PCR-amplification of the 1.2-kbp band from the normal *ADAMTS13* allele produced a 0.5-kbp band from USS-KK3, but not from her mother (KK2). (D) Sequencing of the 1.8- and 0.5-kbp bands in (C) indicated a 1782-bp deletion in USS-W4 and a 729-bp deletion in USS-KK3, respectively.



ment direct sequencing, we selected genomic qPCR (Aldape et al. 2002; Kuramitsu et al. 2012), using primer pairs containing at least one of the two primers used in direct sequencing (Fig. 1B). Combining direct sequencing and genomic qPCR should reveal any defects occurring within or between primer target sequences.

The study protocol was approved by the ethical committee of the National Cerebral and Cardiovascular Center; only subjects who provided written informed consent for genetic analyses were included. This study involved three USS families, USS-W, -X, and -KK (Fig. 2A). Clinical data of the patients (USS-W4, -X5, and -KK3) and the basis of diagnosis were described previously (Fujimura et al. 2011). Plasma ADAMTS13 activities for patients and family members are shown in Figure 2A. No subjects had ADAMTS13 inhibitors. The results of direct sequenc-

ing are also shown in Figure 2A. USS-W4 was a heterozygote with paternal c.1648G>A (p.G550R), USS-X5 had no causative mutations, and USS-KK3 was a heterozygote with maternal c.841T>A (p.C281S). Pathologically unrelated missense polymorphisms (p.Q448E, p.P475S, p.G1181R) (Kokame et al. 2011) were also identified in them (Fig. 2A).

Genomic DNA was prepared from blood and subjected to real-time PCR to quantify the copy numbers of each *ADAMTS13* exon. Each primer pair was designed, using Primer-BLAST (NCBI), to contain at least one of two primers used in direct sequencing (Table S1). A primer pair for the qBiomarker Multicopy Reference Copy Number Assay (MRef, Qiagen, Valencia, CA), which recognizes a stable sequence that appears >60 times throughout the human genome, was used to precisely normalize sample DNA input (~4 ng/reaction). PCR was performed using the QuantiFast SYBR Green PCR Kit (Qiagen) for all regions except exon 7 and the KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) for exon 7. Dimethyl sulfoxide was added (final concentration, 5%) for amplification of exon 8. Fluorescence intensities were detected using the Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA), and each threshold cycle (Ct) value was calculated using the MxPro software (Agilent Technologies).

In genomic qPCR, the difference in Ct among subject DNAs is important information. An increase in Ct value of 1.0 indicates a heterozygous deletion of the target region, whereas a decrease of 0.58 indicates a heterozygous duplication. Ct values of the *ADAMTS13* qPCR indicated that exons 7 and 8 were heterozygously absent in USS-W4 and that exon 27 was heterozygously absent in USS-KK3 (Fig. 2B). By contrast, genomic qPCR revealed no abnormalities in USS-X5.

To confirm the deletions and narrow the deleted regions, we performed PCR using primer pairs specific to regions surrounding the deleted exons. Primers 5′-CACCTCCCCACAGACTCCTA-3′ (intron 6) and 5′-AGGCGGGCAAATCATGAGG-3′ (intron 8) amplified a 3.6-kbp band from the normal allele and a 1.8-kbp band from the mutant allele of USS-W4 (Fig. 2C, *left*). Thus, ~1.8 kbp was deleted within the region straddling exons 7 and 8 in USS-W4. The precise sites where the deletions occurred were determined by sequencing the lower PCR band, which revealed that loss of exons 7 and 8 was caused by a 1782-bp deletion ranging from the 60th nucleotide of exon 7 to the 373rd nucleotide of intron 8 (c.746_987+373del1782) (Figs. 2D, S2A). We confirmed the compound heterozygosity of p.G550R and c.746_987+373del1782 in USS-W4 by genomic PCR of the family members. The patient's mother and brother, but not father, had c.746_987+373del1782 (Fig. 2C, *left*). Direct sequencing indicated that the patient's father, but not mother and brother, had p.G550R (Fig. 2A, *left*).

On the other hand, primers 5′-AGTCACATAGCCA GCAGTGG-3′ (intron 26) and 5′-GCACTGAGCAGAG TGGTCTT-3′ (intron 27) amplified a 1.2-kbp band from the normal allele and a 0.5-kbp band from the mutant allele of USS-KK3 (Fig. 2C, *right*). Thus, ~0.7 kbp was deleted within the region straddling exon 27 in USS-KK3. Sequencing the lower band revealed that loss of exon 27 was caused by a 729-bp deletion ranging from the 36th nucleotide of exon 27 to the 587th nucleotide of intron 27 (c.3751_3892+587del729) (Figs. 2D, S2B). Although the patient's father could not be genetically analyzed, her mother had p.C281S (Fig. 2A, *right*), but not c.3751_3892+587del729 (Fig. 2C, *right*). Thus, it was likely that USS-KK3 was a compound heterozygote of p.C281S and c.3751_3892+587del729.

In conclusion, this study identified two USS patients carrying *ADAMTS13* alleles bearing exon deletions. Extensive defects of *ADAMTS13* may be more common than we expect, and genomic qPCR analysis will be effective for identifying such defects in USS patients. Of the three patients we examined, one did not exhibit abnormalities detectable by either direct sequencing or genomic qPCR. Because these combined analytical methods cannot detect large-scale events such as inversions and translocations that do not affect sequences or copy numbers of target regions, the patient may carry such a defect in *ADAMTS13*. Alternatively, plasma ADAMTS13 deficiency in the patient may be brought about by defects other than *ADAMTS13*, for example, genes involved in synthesis, folding, or secretion of ADAMTS13. Finally, we propose well-designed comprehensive genomic qPCR to complement the weakness of direct sequencing of candidate genes.

Acknowledgments

We thank Ayami Isonishi for her technical assistance. Y. E. was a research resident supported by the Association for Preventive Medicine of Japan at the National Cerebral and Cardiovascular Center. This work was supported in part by grants from the Ministry of Health, Labour, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and from Japan Society for the Promotion of Science.

Conflict of Interest

M. M. is a clinical advisory board for Alexion Pharmaceuticals. Y. F. is a clinical advisory board for Baxter Bioscience and for Alexion Pharmaceuticals.

References

Aldape, K., D. G. Ginzinger, and T. E. Godfrey. 2002. Real-time quantitative polymerase chain reaction: a potential tool for genetic analysis in neuropathology. Brain Pathol. 12:54–66.

Fujimura, Y., M. Matsumoto, A. Isonishi, H. Yagi, K. Kokame, K. Soejima, et al. 2011. Natural history of Upshaw-
Schulman syndrome based on *ADAMTS13* gene analysis in Japan. J. Thromb. Haemost. 9(Suppl. 1):283–301.

Hing, Z. A., T. Schiller, A. Wu, N. Hamasaki-Katagiri, E. B. Struble, E. Russek-Cohen, et al. 2013. Multiple in silico tools predict phenotypic manifestations in congenital thrombotic thrombocytopenic purpura. Br. J. Haematol. 160:825–837.

Kokame, K., and T. Miyata. 2004. Genetic defects leading to hereditary thrombotic thrombocytopenic purpura. Semin. Hematol. 41:34–40.

Kokame, K., M. Matsumoto, K. Soejima, H. Yagi, H. Ishizashi, M. Funato, et al. 2002. Mutations and common polymorphisms in *ADAMTS13* gene responsible for von Willebrand factor-cleaving protease activity. Proc. Natl. Acad. Sci. USA 99:11902–11907.

Kokame, K., Y. Kokubo, and T. Miyata. 2011. Polymorphisms and mutations of *ADAMTS13* in the Japanese population and estimation of the number of patients with Upshaw-Schulman syndrome. J. Thromb. Haemost. 9:1654–1656.

Kuramitsu, M., A. Sato-Otsubo, T. Morio, M. Takagi, T. Toki, K. Terui, et al. 2012. Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia. Blood 119:2376–2384.

Levy, G. G., W. C. Nichols, E. C. Lian, T. Foroud, J. N. McClintick, B. M. McGee, et al. 2001. Mutations in a member of the *ADAMTS* gene family cause thrombotic thrombocytopenic purpura. Nature 413:488–494.

Lotta, L. A., I. Garagiola, R. Palla, A. Cairo, and F. Peyvandi. 2010. *ADAMTS13* mutations and polymorphisms in congenital thrombotic thrombocytopenic purpura. Hum. Mutat. 31:11–19.

Matsumoto, M., K. Kokame, K. Soejima, M. Miura, S. Hayashi, Y. Fujii, et al. 2004. Molecular characterization of *ADAMTS13* gene mutations in Japanese patients with Upshaw-Schulman syndrome. Blood 103:1305–1310.

Schouten, J. P., C. J. McElgunn, R. Waaijer, D. Zwijnenburg, F. Diepvens, and G. Pals. 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 30:e57.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer pairs for *ADAMTS13* genomic qPCR.

Figure S1. Combinatorial analysis of direct sequencing and genomic qPCR should catch any defects occurring on and between the primer target sequences. Direct sequencing detects mutations such as point mutations (including substitutions, insertions, and deletions), short insertions, and deletions in the exons and exon–intron boundaries (A), but misses mutations on the allele that contains no or mismatched primer target sequences (B). Genomic qPCR for quantifying the copy numbers of target regions complements the results of direct sequencing.

Figure S2. Deleted regions and flanking sequences of *ADAMTS13* identified in two patients with USS. The 1782- and 729-bp regions (red letters) were deleted in patients USS-W4 (A) and USS-KK3 (B), respectively. Lowercase and uppercase sequences indicate introns and exons, respectively. Underlined sequences adjacent to the breakpoints may cause microhomology-mediated end joining (MMEJ) (McVey and Lee. Trends Genet. 2008;24:529–538).

I. 易血栓傾向とは？

1. 先天性血栓性素因

国立循環器病研究センター分子病態部部長 宮田 敏行(Miyata, Toshiyuki)
金沢大学医薬保健研究域保健学系病態検査学准教授 森下英理子(Morishita, Eriko)

THROMBOSIS and Circulation

§論文のポイント

[1] 先天性血栓性素因として，遺伝性のアンチトロンビン欠損症，プロテインC欠損症，プロテインS欠損症が知られている．いずれもヘテロ接合体が先天性血栓性素因となる．

[2] アンチトロンビン，プロテインC，プロテインSはいずれも血管内皮細胞上で抗凝固能を発揮し血液の流動性維持に働いている．

[3] 最近，プロトロンビン遺伝子に機能獲得変異であるArg596Leu変異を持ち，アンチトロンビン抵抗性を示す血栓症家系が本邦で報告され，世界的に注目を集めている．

[4] 遺伝子解析に多用されるPCR-DNAシークエンス法は，小さい変異は同定できるが，欠失などの大きな変異の同定は難しい．変異が同定できなかったとしても，遺伝子欠失などが生じている可能性を考慮する必要がある．

§キーワード

静脈血栓塞栓症／アンチトロンビン欠損症／プロテインC欠損症／プロテインS欠損症／遺伝子変異

血栓性素因とは

血流の流動性はむやみに血栓ができないよう，血栓制御機構により保たれており，血栓を生じさせる能力とそれを制御し阻止する能力のバランスの上に成り立つ．150年以上前にVirchow博士は，血栓は血流・血管壁・血液成分の異常により生じると述べ，今日でもこの考えは支持されている．血栓性素因とは血栓を生じやすい傾向とされ，先天性と後天性に分類される．先天性血栓性素因としては，遺伝性のアンチトロンビン(AT)欠損症，プロテインC(PC)欠損症，プロテインS(PS)欠損症が知られている[1)-3)]．日本における発症頻度は，一般住民を対象とした報告によるとPC欠損症0.13%，AT欠損症0.15%と欧米人と差はないが，PS欠損症は1.12%と5～10倍高いのが特徴である．特に，PS分子異常症であるPS Tokushima変異(PS Lys196Glu変異)ヘテロ接合体は一般住民の約55人に1人認められ，日本人の遺伝子多型と考えられる．白人種には凝固V因子Leiden変異とプロトロンビンG20210A変異も先天性血栓性素因として知られているが，これらは日本人を含む東アジア人にはみられない．日本人の約4%にみられるプラスミノーゲン異常症(Ala601Thr変異)は静脈血栓塞栓症患者に高率にみられるものではないので，先天性血栓性素因と考えられていない[4)]．

先天性血栓性素因の保有者は若年(40～45歳より前)に血栓を発症し，しばしば血栓は再発し家族歴を示すことが多い．血栓は静脈血栓塞栓症や肺塞栓などが主であるが，腸間膜静脈や脳静脈にもまれに生じる．AT，PC，PSはいずれも血管内皮細胞上で抗凝固能を発揮し血液の流動性維持に働いている．血栓は手術，妊娠，長期臥床，悪性腫瘍などの環境因子や薬剤(ホルモン補充療法や経口避妊薬)の使用などの環境因子が重なることにより発症することが多い[5)]．

凝固カスケード反応

凝固カスケード反応では，組織因子が最も重要な血栓惹起因子である．組織因子は，血液に接する細胞(血球系細胞，血小板，血管内皮細胞)に通常発現していない．組織因子は血管外膜の繊維芽細胞，中膜の血管平滑筋細胞に恒常的に発現し血管損傷の際の止血に働く．また，粥状硬化巣に集積する単球マクロファージも組織因子を発現し，粥腫巣の破綻に伴い凝固因子を活性化する．

血液中には活性型Ⅶ(Ⅶa)がⅦの0.5-1%程度存在し，これが組織因子に結合することによりⅦa-組織因子複合体が形成され，Ⅸ活性化能とX活性化能を発揮する(凝固開始反応と呼ぶ)(図1)．このⅦaはわずかな量なので，凝固の開始時に形成されるⅦa-組織因子複合体もわずかであり，こうして生成したXaからは少量のトロンビンしか生成しない．しかし，少量のトロンビンは凝固の補助因子であるⅧとVを活性化させる．活性型であるⅧaとVaが生成されると，Xやプロトロンビンの活性化が効率よく進行する．更に，トロンビンはXIを活性化し凝固反応を促進する．このトロンビンによるXI活性化反応は生理的にも重要であると考えられている．また，Ⅶa-組織因子複合体はⅦの活性化も行い，これにより凝固がさらに進行することになる．これらの活性化反応が進んだ結果，大量のトロンビンが生成する(凝固増幅反応と呼ぶ)[6)]．

トロンビンは多機能プロテアーゼである．フィブリノーゲンのフィブリンへの変換やトランスグルタミナーゼであるXⅢの活性化を行うだけでなく，血小板膜上のトロンビン受容体protease activated receptor(PAR)を活性化することにより血小板の活性化を行う(図1)[6)]．トロンビンにより活性化された血小板は濃染顆粒からADPの放出を行い，血小板インテグリンの活性化が起こるとともに，濃染顆粒からポリリン酸が放出され，内因系凝固反応を活性化させる．このポリリン酸は，トロンビンやXaによるV活性化を促進するなど，多彩な活性を示し，凝固系に極めて重要な役割を果たしていると報告されている[7)]．

血管内皮細胞上の抗凝固機構

血管内皮細胞上の抗凝固機構は，内皮細胞上のヘパラン硫酸グリコサミノグリカン上での反応(ヘパラン硫酸依存性抗凝固機構)とPC抗凝固機構がある(図2)．

血管内皮細胞はヘパリン様の構造を持つヘパラン硫酸プロテオグリカン(heparan sulfate proteoglycan：HSPG)をもつ．このヘパラン硫酸

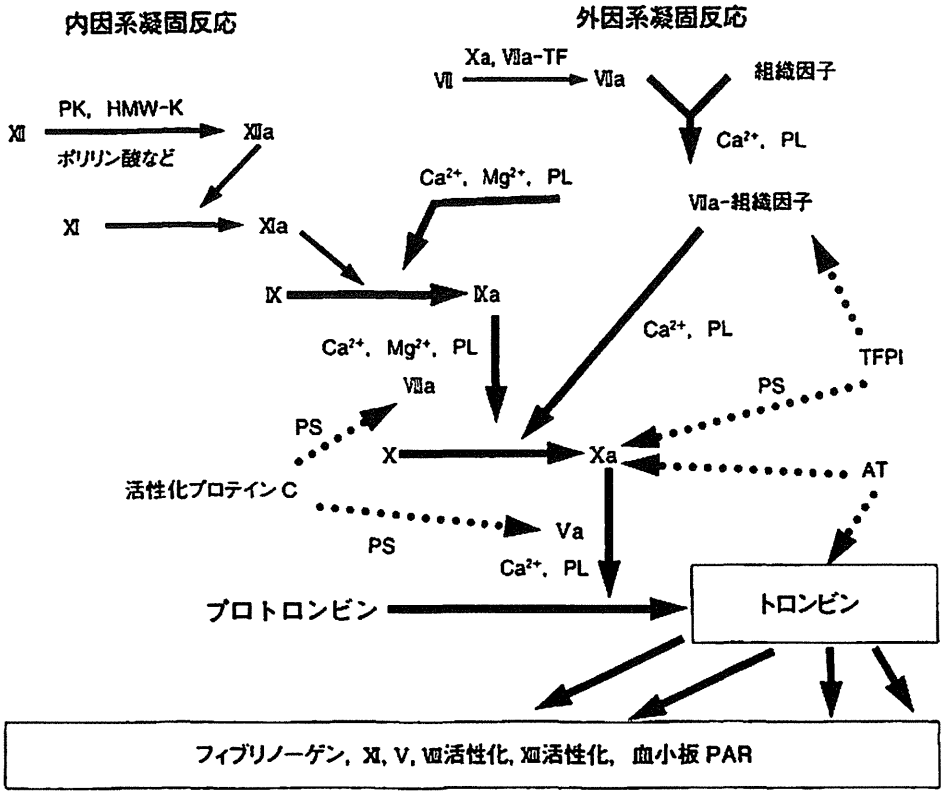


図1　凝固カスケード反応と制御因子の作用点

にATや組織因子経路インヒビター(Tissue factor pathway inhibitor：TFPI)が結合し，トロンビンやXaを効率良く阻害することにより，凝固反応を抑制している．一方，内皮細胞は1回膜貫通蛋白質であるトロンボモジュリンを恒常的に発現している．トロンビンはトロンボモジュリンに結合すると，フィブリノーゲンのフィブリン変換能が極度に低下しPC活性化能を獲得する．この際，1回膜貫通蛋白質である内皮細胞PC受容体(Endothelial cell protein C receptor：EPCR)がPCを内皮細胞表面に濃縮させ，トロンボモジュリンに結合したトロンビンによる活性化反応を促進させる(図2)．この反応で生じた活性化PC (Activated protein C：APC)は，活性型V (Va)と活性型Ⅷ (Ⅷa)を限定分解により不活性型へと変換することにより凝固反応を抑制する．

このように血管内皮細胞は強い抗血栓能を示し，血液の流動性に大きく寄与している．図2に登場する抗凝固因子のうち，AT，PC，PSの遺伝子異常による欠損症はしばしば静脈血栓塞栓症患者にみられることから，先天性血栓性素因として知られている[1-3]．TFPI，トロンボモジュリン，EPCRの遺伝子異常は静脈血栓塞栓症患者に散発的にみられる程度であり，血栓性素因と考えられていない．最近，トロンボモジュリンのミスセンス変異が非典型溶血性尿毒素症候群にみられ，遺伝的素因であるとの報告がなされた[8]．

AT欠損症

ATはセリンプロテアーゼインヒビター(セルピンと総称される)で，トロンビンに加え，Xa，Ⅸa，Ⅶa-組織因子複合体，Ⅺa，Ⅶaを阻害する[1]．ATの血漿中濃度は2.4 μmol/Lであり，プロトロンビン(血漿中濃度，1.4 μmol/L)より豊富に循環している．血中半減期は2.5-3.0日である．トロンビンなどのプロテアーゼと1：1で複合体を形成し活性を失活させる．ATによるプロテアーゼ阻害はヘパリンやヘパラン硫酸により増強され，ヘパリンはプロテアーゼ阻害を1万倍程度にまで加速

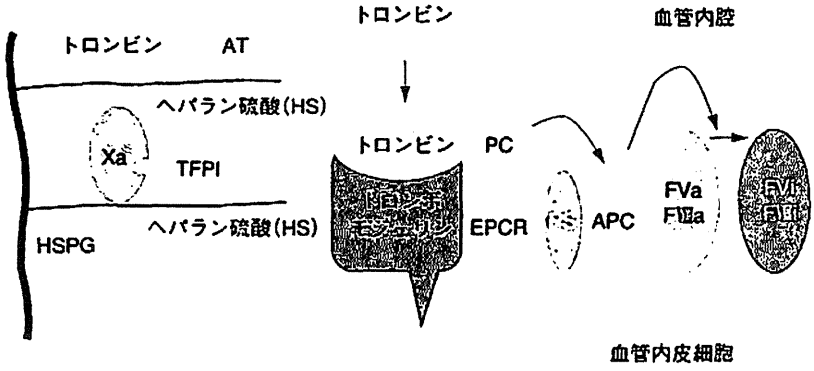


図2　血管内皮細胞上の抗血栓蛋白質群

させる．このようにヘパリンの抗凝固作用はATを介して発揮される．

AT欠損症は，活性と抗原が共に低下するⅠ型欠損症と，活性は低下するものの抗原量は正常値を示すⅡ型異常症に分類される．Ⅱ型異常症は，❶反応部位に障害をもつもの，❷ヘパリン結合部位に障害をもつもの，❸ヘパリン結合部位と反応部位の両方に障害をもつ多面的なものに分けられる．血漿中のAT活性はヘパリン存在下でXa活性もしくはトロンビン活性の阻害能を合成基質を用いて測定する．この測定法はヘパリン依存性のATを測定しているため，Ⅱ型異常症を更に3種のタイプに分けることは実質的に難しい．

日本人の一般住民を対象にヘパリン依存性のAT活性を測定した研究では，約650人に1人が欠損症と考えられる低活性を示した[9]．この頻度はイギリスで得られたものとほぼ同じであった．日本で行われた静脈血栓症患者群と一般住民群にみられるAT欠損症の頻度の比較から，AT欠損症は静脈血栓塞栓症の強いリスクであった．これをⅠ型とⅡ型に分けてみると，AT欠損症Ⅰ型は極めて強い血栓症のリスクであったが，Ⅱ型はそれほど強いリスクを示さなかった[10]．

PC欠損症

PCはビタミンK依存性因子であり，軽鎖はGlaドメインと2つのEGF様ドメインから成り，重鎖は活性化ペプチドにつづいてセリンプロテアーゼドメインをもつ．血漿濃度は65 nmol/L．血中半減期は6～8時間とかなり短い．抗凝固薬ワルファリンはビタミンKエポキシド還元酵素を阻害しGla残基の形成を抑制するため，ワルファリンを服薬するとプロトロンビン等の凝固因子だけでなく，PCやPSといった凝固制御因子も低下する．このため，血栓性素因を疑う症例でのPCやPSの活性・抗原量の測定は，ワルファリン投与前の検体で行う必要がある．また，PCの血中半減期はプロトロンビンなどの他のビタミンK凝固因子の半減期より短いため，先天性PC欠損症では，急性期のヘパリン類による治療からワルファリンに切り換える際にPCが低下し皮膚壊死を起こす可能性がある．ヘパリン併用下にワルファリンを少量から治療域に導き，安定した後にヘパリンを中止する必要がある．

EPCRに結合したPCは血管内皮細胞上でトロンビン-トロンボモジュリン複合体によりAPCに活性化され，APCがVaとⅧaを分解不活化することにより，凝固カスケード反応を制御するが，これに加えて，APC-EPCR複合体は内皮細胞のPARを活性化し内皮細胞に保護的に働く[11]．また，APCはアポトーシスを起こした細胞から放出されるヒストンを切断し，ヒストン依存性の細胞死を減弱させる機能を持つと報告されている[12]．

PC活性は凝固時間法と合成基質法で測定できる．多くのPC欠損症では両活性がともに低下するが，Gla領域に変異を認める例では凝固時間法のみが低値を示す．PCの抗原量を求め，活性と抗原が共に低値を示す例をⅠ型欠損症，活性のみ低値を示し抗原量は正常値を示す例をⅡ型欠損症(異常症)と呼ぶ．

PS欠損症

PSは本因子が精製された米国シアトル市にちなんで命名された．本因子も生合成にビタミンKを要求する．血中半減期は42時間である．PSは抗凝固機能と細胞保護作用を示す多機能蛋白質である．抗凝固機能はAPC依存性とTFPI依存性がある．

PSはAPCによるⅧaとVaの分解・不活化を促進することにより抗凝固機能を発揮する．PSの血中濃度は300 nmol/Lであり，約60%のPSはC4b結合蛋白質(C4BP)と1：1で結合して血中を循環している．残りの約40%は遊離型として循環している．C4BPの約80%は6～7本のα鎖に1本のβ鎖がジスルフィド結合で繋がった分子(C4BPβ＋)であるが，残りの約20%はβ鎖をもたない．β鎖を含むC4BPβ＋はPSと1：1で複合体(PS-C4BP複合体)を形成し血中に循環する．この複合体中のPSはAPCコファクター活性，すなわち抗凝固活性が極めて低い．PSの抗凝固活性は主に遊離型PSが担う．PSはTFPIによるXa活性の阻害を促進する作用を示し，外因系凝固反応を抑制する抗凝固機能も示す[13]．

PSの細胞保護作用は，分子のC末端領域の性ホルモン結合グロブリン様ドメインを介して細胞膜上の3種のチロシンキナーゼ型受容体Tyro3, Axl, Mer（頭文字をとってTAM受容体とも呼ばれる）に結合することにより発揮される．

PS活性はAPCのコファクター活性を凝固時間法で測定できる．PS抗原量は総抗原量，遊離型抗原量，C4BP結合型抗原量の3通りを測定できる．一般的に遊離型PS抗原量はPS活性と一致するが，異常分子では乖離する例も報告されている．活性と抗原量が共に低値を示す例をⅠ型欠損症，活性のみ低値を示し抗原量は正常値を示す例をⅡ型欠損症(異常症)とよぶ．PS活性測定で最も注意すべき点は，妊娠時の生理的変動として活性が低下する点である．正常妊婦でも30%程度まで低下する場合があり，欠損症との鑑別が困難である．

遺伝子解析結果の解釈

近年の遺伝子解析技術の向上により，先天性血栓性素因の遺伝子解析が世界的に進められ，多くの遺伝子変異が同定されている．AT欠損症，PC欠損症，PS欠損症の遺伝子解析は，本邦でも広く進められている[14]．広く行われている遺伝子解析法は，主にエクソン領域をPCR法で増幅し，塩基配列をDNAシークエンサーで解読する手法をとる．このため，次のような手法の限界があるので注意を払う必要がある．

PCR-DNAシークエンス法はアミノ酸変異を生じるミスセンス変異，停止コドンに変化するナンセンス変異，数塩基の欠失・挿入によるフレームシフト変異，スプライスのコンセンサス配列に生じる変異といった小さい変異を同定することができる．しかし，本法ではエクソンや遺伝子全体の欠失といった大きな遺伝子上の異常を同定することは難しい．また，重症血友病A患者に広くみられるエクソンの逆位も同定できない．PCR-DNAシークエンス法で変異が同定できなかったとしても，遺伝子欠失などが生じている可能性を考えておくことも必要である．ミスセンス変異では，それが蛋白質の機能を損なうかどうかを詳しく調べる必要がある．このように，活性や抗原量の低下を示す患者の遺伝子解析の結果が得られたとしても，その遺伝子変異を慎重に解釈する必要がある．

新しい血栓性素因：アンチトロンビン抵抗性

最近，アンチトロンビン抵抗性という新しい血栓性素因が本邦の血栓症を示す家系から報告された．この家系では，プロトロンビン遺伝子にArg596Leu変異をもち，凝固活性は少し低下するが，アンチトロンビンによる活性阻害を受けない機能獲得変異であった[15]．本変異保有者は今のところこの家系に限られているが，今後より詳細な研究により，その頻度などが明らかになり，日本人の静脈血栓塞栓症の発症にどの程度関わるかが明らかになるだろう．

References

1) De Stefano V, Finazzi G, Mannucci PM：Inherited thrombophilia：pathogenesis, clinical syndromes, and management. Blood 87：3531-3544, 1996
2) Lane DA, Mannucci PM, Bauer KA, et al：Inherited thrombophilia：Part 2. Thromb Haemost 76：824-834, 1996
3) Lane DA, Mannucci PM, Bauer KA,