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Cutoff Values of D-Dimer and FDP in Plasma for the Diagnosis of Thrombosis

Toshihiro Kaneko[1], Hideo Wada[2,*], Katsuya Onishi[2], Yasunori Abe[3], Satoshi Ota[4], Norikazu Yamada[4], Takeshi Matsmoto[5], Naoyuki Katayama[5], Akihiro Sudou[6], Atsumasa Uchida[6] and Tsutomu Nobori[2]

[1]*Patient Safety Division, Departments of* [2]*Molecular and Laboratory Medicine,* [3]*Central Laboratory,* [4]*Cardiology,* [5]*Hematology and* [6]*Orthopaedic Surgery, Mie University Graduate School of Medicine, Tsu, Japan*

Abstract: Fibrin-related markers, such as fibrin and fibrinogen degradation products (FDP) and D-dimer, are considered useful for the diagnosis of thrombosis. However, the evidence for making a diagnosis of thrombosis based on fibrin-related markers is still not yet well established.

The plasma concentrations of soluble fibrin and D-dimer were prospectively measured in 680 inpatients suspected of having thrombosis between October 1, 2003 and January 31, 2005, and correlated with thrombosis.

The normal ranges of D-dimer and FDP were within 0.76 μg/ml and 1.50 μg/ml, respectively. Out of 680 patients, 129 patients showed plasma concentrations associated with thrombosis, including 73 with deep venous thrombosis (DVT)/ pulmonary embolism (PE). The plasma D-dimer and FDP concentrations were significantly higher in the patients with thrombosis than in the patients without thrombosis, but there were no significant differences in the D-dimer and FDP levels among the patients with thrombosis. The plasma D-dimer levels were significantly correlated with the plasma FDP levels in all the patients and there was no significant difference in the ratio of FDP/ D-dimer among the various diseases. A ROC analysis showed that both FDP and D-dimer were useful for the diagnosis of all types of thrombosis and DVT. The cutoff values of D-dimer (3.8 μg/ml) and FDP (7.7 μg/ml) had high sensitivity, specificity and negative predictive values (NPV) but low positive predictive value.

Our findings suggest that FDP showed a close correlation with D-dimer, which is known to be a marker for a hypercoagulable state, and it is also reflects a high risk for thrombosis.

Keywords: Hypercoagulable state, deep vein thrombosis, fibrin and fibrinogen degradation products, D-dimer.

INTRODUCTION

Fibrin and fibrinogen degradation products (FDP) including D-dimer, are considered to be useful for detecting the state of thrombosis, and they have been reported to be elevated in deep vein thrombosis (DVT)/pulmonary embolism (PE) [1-3], disseminated intravascular coagulation (DIC) [4, 5], acute myocardial infarction (AMI) [6, 7] and thrombotic thrombocytopenic purpura (TTP) [8]. However, a serum assay of FDP can be rather time consuming since it is necessary to wait for clot formation and clot lysis. Recently, the FDP levels in plasma were reported to be successfully measured using a specific monoclonal antibody [9], thus making the plasma FDP assay as fast and easy perform as the D-dimer assay. The International Society of Thrombosis and Haemostasis (ISTH) has established the diagnostic criteria for overt-DIC using fibrin-related markers; FDP, D-dimer and soluble fibrin [10]. D-dimer is widely used to diagnose thrombosis as DVT but many of the commercially available D-dimer assay kits contain different monoclonal antibodies, standard substances and they are based on different assay systems. Several studies [11, 12] have reported basic data for the standardization of D-dimer assays; however, this issue remains to be resolved.

PE is a common, frequently undiagnosed, and potentially fatal cause of several common symptoms, including dyspnoea and chest pain [13-15]. Since PE is often a fatal disease caused by DVT, an early clinical evaluation of DVT [16] and PE [17] is therefore crucial. In this regard, D-dimer has been reported to be a negative predictor for DVT and a D-dimer level of less than 0.5 μg/ml is considered to exclude DVT/PE with the most commonly used D-dimer assays in Europe and North America [16]. DIC [18, 19] is often observed in patients with leukaemia, solid cancers, infections, gynaecological conditions and aneurysms, and it is also frequently associated with severe bleeding and organ failure. Since DIC is a fatal condition [20], it is important to diagnose it early using hemostatic molecular markers [21].

The present study was designed to evaluate the cutoff values of FDP, including D-dimer, in the diagnosis of several types of thrombosis, including DVT, DIC, cerebral thrombosis, and AMI, prospectively. For this purpose, we determined the plasma concentrations of these molecules in 680 patients suspected of having thrombosis, as well as in 100 healthy volunteers.

*Address correspondence to this author at the Department of Laboratory Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu-city, Mie-ken 514-8507, Japan; Tel: 81-59-232-1111; Fax: 81-59-231-5204; E-mail: wadahide@clin.medic.mie-u.ac.jp

1567-2700/08 $55.00+.00

MATHERIALS AND METHODS

Subjects

From October 1, 2003 to January 31, 2005, 680 patients (age, 60.2 ±17.3 years, mean ± SD; 411 females and 269 males) were suspected of having thrombosis (DVT or DIC) in the hospitals affiliated with Mie University School of Medicine. Plasma concentrations of FDP and D-dimer were examined in these patients and correlated with thrombosis. The study protocol was approved by the Human Ethics Review Committees of the participating institutions and a signed consent form was obtained from each subject. Thirty-five patients within 7 days after operation (OPE) and 28 patients who had undergone liver transplantation (LT) were excluded from analysis of the cutoff value. Among the remaining 617 patients, 488 patients (56.8 ±17.9 years; 279 females and 209 males) did not have any thrombosis, while 129 patients had thrombotic diseases [73 with DVT (62.7 ±17.8 years; 60 females and 13 males) including 30 with DIC (64.7 ±15.1 years; 10 females and 20 males), 12 with cerebral thrombosis (71.0 ±2.9 years; 5 females and 7 males); 6 with acute myocardial infarction (AMI) (68.1 ±11.5 years; 2 females and 4 males), 4 with arteriosclerosis obliterans (ASO) (71.2 ±7.0 years; 2 females and 2 males) and 4 with portal vein thrombosis (63.1 ±11.3 years; 2 females and 2 males)] (Table **1**). DVT was diagnosed with Doppler ultrasonographic examination or venography. DIC was diagnosed using the ISTH overt-DIC diagnostic criteria [10]. Cerebral thrombosis was diagnosed by either computed tomography (CT) or magnetic resonance imaging (MRI) and AMI was diagnosed by the electrocardiogram and laboratory data. Portal vein thrombosis PVT was diagnosed by Doppler ultrasonographic examination or CT. Among the underlying diseases in these patients, cancer was identified in 184 patients, orthopaedic conditions in 152, cardiovascular diseases in 66, digestive diseases in 39, infectious diseases in 33, autoimmune diseases in 31, hematological diseases in 23, diabetes mellitus in 15, obstetrics in 15, thrombophilia in 10, trauma and burns in 9, and no underlying diseases in 31 (Table **1**).

Citrated blood samples were obtained from the peripheral veins of healthy subjects (see below) and patients under fasting conditions and then centrifuged for 20 min at 3,000 rpm. The supernatants (plasma) were analyzed within 4 h. Plasma concentrations of FDP and D-dimer were measured in patients with thrombosis at the onset and those without thrombosis at the first consultation. The same parameters were also measured in 100 healthy subjects (mean age, 41.5 years; range, 20 - 58 years; 47 males and 53 females), who were free of any diseases including thrombotic disease or hyperlipidemia as confirmed by an annual medical check-up.

Measurement of Plasma Concentrations of D-Dimer, FDP, Antithrombin, Plasmin Inhibitor and Plasminogen

The plasma D-dimer and FDP levels were measured by the latex agglutination method using the Nanopia D-dimer and Nanopia FDP (Daiichi Kagakuyakuhin, Tokyo, Japan). The activities of antithrombin, plasmin inhibitor and plasminogen were measured by the chromogenic substrate method using a testchyme ATIII 2 kit, testchyme APL 2 kit and testchyme PLG 2 kit (Daiichi Kagakuyakuhin), respectively.

Table 1. Underlying Diseases of Subjects

	DVT/PE	DIC	CT	AMI	ASO	PVT	TH(-)	Total
Orthopaedic C	24	1		1			126	152
Solid organ cancer	11	7	2			3	161	184
Digestive D	3					1	35	39
Cardiovascular D	3	5	1	4	1		52	66
Infectious D		12					21	33
Without underlying D	22	2	6	1			0	31
Autoimmune D	1						30	31
Hematological D	2	2					19	23
Diabetes mellitus	1		1		3		10	15
Obstetrics	2						13	15
Thrombophilia	3		2				5	10
Trauma/burn	1	1					7	9
Others							9	9
Total	73	30	12	6	4	4	488	617

C: conditions, D: diseases, DVT: deep vein thrombosis, PE: pulmonary embolism, DIC: disseminated intravascular coagulation, CT: cerebral thrombosis, AMI: acute myocardial infarction, ASO: arteriosclerosis obliterans, PVT: portal vein thrombosis, TH(-): without thrombosis.

Statistical Analysis

All data are expressed as the mean ± SD. The differences between the groups were examined for statistical significance using Mann-Whitney's U test while correlation between 2 variables was tested by Pearson's correlation analysis. A *P* value less than 0.05 denoted a significant difference. The usefulness of D-dimer and soluble fibrin (SF) for the diagnosis of thrombosis and DVT was examined based on a receiver operating characteristic (ROC) analysis [22]. The cutoff values were determined by the ROC analysis. All statistical analyses were performed using SPSS II software package (SPSS Japan, Tokyo).

RESULTS

DVT or PE was observed in various diseases, although the frequency of DVT or PE was markedly high in the patients with orthopaedic diseases and solid cancer. Meanwhile, the frequency of DIC was high in the patients with infectious diseases and solid cancers (Table **1**). In the healthy subjects, the plasma concentrations of D-dimer and FDP were not distributed normally, with maximum values of 1.16 μg/ml and 2.40 μg/ml, minimum values of 0.25 μg/ml and 0.50 μg/ml, and median values of 0.48 μg/ml and 0.90 μg/ml, respectively. In the healthy volunteers, the 95% confidence intervals (CI) of D-dimer and FDP were 0.76 μg/ml and 1.50 μg/ml, respectively.

The plasma D-dimer and FDP concentrations (median; 25 % - 75% tile) were significantly higher in the patients with thrombosis (9.45 μg/ml; 4.15 – 14.94 μg/ml and 14.77μg/ml; 7.99 – 23.34 μg/ml), OPE (7.13 μg/ml; 2.89 – 11.94 μg/ml and 11.10 μg/ml; 6.01 – 18.14 μg/ml) or LT (6.88 μg/ml; 2.37 – 10.77 μg/ml and 11.28 μg/ml; 4.58 – 16.53 μg/ml) than in the patients without thrombosis (1.09 μg/ml; 0.74 – 2.29 μg/ml and 2.17 μg/ml; 1.46 – 4.64 μg/ml)($p< 0.001$, respectively). The plasma D-dimer and FDP concentrations were also significantly higher in the patients without thrombosis than in the healthy volunteers ($p< 0.001$) (Fig. (**1**)). However, there were no significant difference in D-dimer and FDP levels among the patients with thrombosis, those with OPE and those with LT, and among various underlying diseases. The plasma levels of antithrombin (AT), plasminogen and plasmin inhibitor activity were significantly lower in the patients with thrombosis than in the patients without thrombosis, and the plasma levels of plasminogen and plasmin inhibitor activity were significantly lower in the patients with LT than in those without thrombosis ($p< 0.01$, respectively) (Table **2**).

The plasma D-dimer and FDP concentrations were significantly higher in the patients with DIC (9.90 μg/ml; 5.30 – 17.50 μg/ml and 15.00 μg/ml; 8.60 – 36.40 μg/ml) and DVT (10.1 μg/ml; 4.95 – 16.35 μg/ml and 17.30 μg/ml; 8.30 – 26.73 μg/ml) than in the patients without thrombosis ($p< 0.01$, respectively) (Fig. (**2**)). The plasma D-dimer and FDP concentrations were higher in the patients with cerebral thrombosis (CT) (4.70 μg/ml; 1.55 – 9.65 μg/ml and 8.00 μg/ml; 4.00 – 15.90 μg/ml) and ASO (9.80 μg/ml; 7.80 – 16.40 μg/ml and 15.20 μg/ml; 8.60 – 18.70 μg/ml) than in the patients without thrombosis ($p< 0.05$, respectively).

The plasma D-dimer levels were significantly correlated with the plasma FDP levels in all patients ($Y = 0.489 X + 0.525$, $R= 0.962$, $p< 0.001$) (Fig. (**3**)). There was no significant difference in the ratio of FDP/DD among the various diseases. In the patients with more than 10.0 μg/ml, 97 patients had more than 70% of plasminogen levels and 78 patients had less than 70% of plasminogen levels. The former was considered the normal fibrinolysis group and the latter was the hyper fibrinolysis group. There was no significant difference in the ratio of FDP/D-dimer between the normal fibrinolysis group (1.64; 1.51 - 1.97) and the hyper fibrinolysis group (1.55; 1.46 - 1.79).

Fig. (**4**) shows the positive predictive values (PPV) for several cutoff values of D-dimer and FDP in the patients with thrombosis. When a D-dimer value of >3.0 μg/ml and an FDP value of >6.0 μg/ml was used, more than 50% of patients, excluding those with liver transplantation or post-operation, had some thrombosis.

An ROC analysis showed that both FDP and D-dimer were useful for the diagnosis of all types of thrombosis and, in particular, DVT (Fig. (**5**)). The areas under the curve (AUC) of D-dimer were similar to that of FDP in all types of thrombosis and DVT/PE. The ROC analysis provided adequate cutoff values of D-dimer and FDP for the diagnosis of all types of thrombosis and DVT/PE (Table **3**). The cutoff values of D-dimer (3.8 and 3.4 μg/ml) for the diagnosis of all types of thrombosis and DVT/PE were similar, while those of FDP (7.6 and 7.7 μg/ml) were also similar. Both D-dimer (3.8 μg/ml) and FDP (7.7 μg/ml) had high sensitivity, specificity and negative predictive value (NPV) but low positive predictive value.

DISCUSSION

In our study, the frequency of DVT or PE was the highest among the various types of thrombosis and DVT or PE was frequently observed in orthopaedic diseases and solid organ cancer, while the frequency of DIC was high in infectious diseases and solid organ cancers. These findings are similar to previous reports [13, 14, 19, 23]. The frequency of thrombosis depends on the underlying disease. Regarding the underlying diseases that are frequently associated with thrombosis, the risk for thrombosis should be evaluated by a simple test such as D-dimer and FDP.

In the present study, the normal ranges of D-dimer and FDP were within 0.76 μg/ml and 1.50 μg/ml, respectively. There are many D-dimer assay kits and the cut off value depends on the kit used. In the most commonly used D-dimer assay in Europe and North America, D-dimer concentrations of less than 0.5 μg/ml are considered to exclude DVT/PE [6]. However, in Japan, the D-dimer concentration is more than 0.5 μg/ml in many patients without thrombosis and this cutoff value is therefore not useful as a NPV for DVT/PE in Japan [24], especially because the D-dimer kits that are frequently used in Japan have a wide normal range (about 0.3 – 2.5 μg/ml). The plasma D-dimer and FDP concentrations were also significantly higher in the patients without thrombosis than in the healthy volunteers, suggesting that some underlying diseases may increase in FDP and D-dimer levels without causing thrombosis.

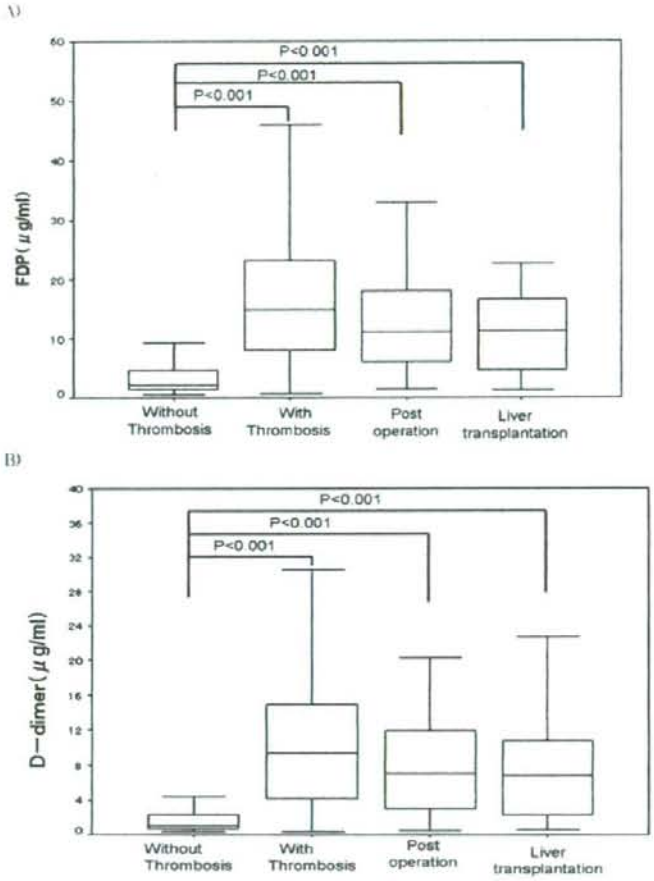

Fig. (1). The plasma levels of FDP and D-dimer in patients with or without thrombosis, either post-operation or after a liver transplantation. A) FDP, B) D-dimer.

Table 2. Plasma Levels of Antithrombin, Plasminogen and Plasmin Inhibitor

	Without TH	With TH	Post Operation	LT
Antithrombin (%)	96.8 (86.5 - 107)	85.3** (73.8 - 98.2)	90.5* (80.3 - 97.2)	88.1* (62.1 - 104.8)
Plasminogen (%)	99.0 (88.0 – 109.6)	90.7** (72.9 – 105.2)	88.8* (79.4 - 103.9)	52.4** (41.4 - 87.0)
Plasmin inhibitor (%)	104.0 (95.0 – 113.3)	96.9** (85.0 – 108.2)	97.3** (90.3 - 105.2)	76.3** (61.3 - 92.6)

Data are shown as median (25 % tile – 75 % tile).
**; $p< 0.01$, *; $p< 0.05$ in comparison to without TH.
TH: thrombosis, LT: liver transplantation.

Although the plasma D-dimer and FDP concentrations were significantly higher in the patients with thrombosis than in the patients without thrombosis, there were no significant differences in the D-dimer and FDP levels among the patients with thrombosis, those who were post-operation and those with LT, thus suggesting that these assays may be useful for patients on medication alone or for pre-operative patients. In the present study, we demonstrated that the concen-

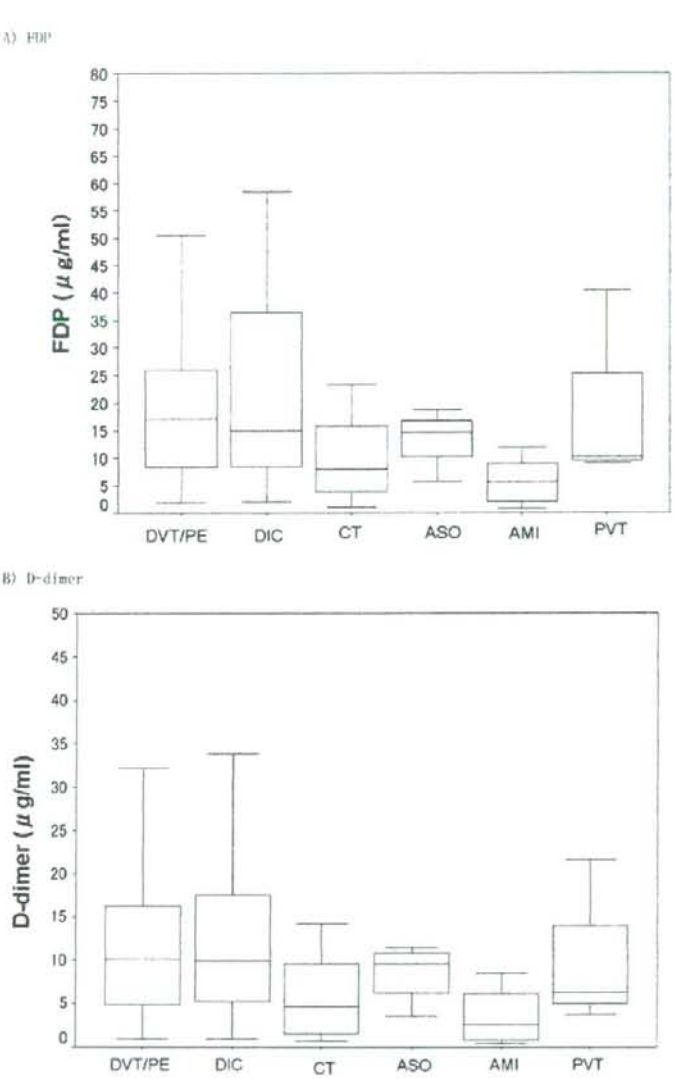

Fig. (2). The plasma levels of FDP and D-dimer in patients with various types of thrombosis.
A) FDP, B) D-dimer.
DVT: deep vein thrombosis, PE: pulmonary embolism, DIC: disseminated intravascular coagulation, CT: cerebral thrombosis, ASO:
AMI: acute myocardial infarction, PVT: portal vein thrombosis, TH: thrombosis, **: $p< 0.01$, *: $p< 0.05$.

trations of both D-dimer and FDP were significantly high in the patients with thrombosis, such as DVT/PE, DIC, CVA and AMI. Therefore, high concentrations of D-dimer and FDP could be considered as markers of thrombosis, since both parameters have also been reported to be elevated in DVT [25, 26], DIC [4, 27] and hyperlipidemia [28]. We previously reported prospective studies that evaluated the soluble fibrin (SF) and D-dimer assay and the cutoff value of the diagnosis for thrombosis [23]. These findings were similar to those of previous reports [23].

The plasma D-dimer levels were significantly correlated with the plasma FDP levels in all patients. It has been reported that the positive rate of FDP for thrombosis depends on the plasma levels of D-dimer [29]. There was no significant difference in the ratio of FDP/DD among the various diseases and between the normal fibrinolysis group and the hyper fibrinolysis group. It has also been reported that there is a hyperfibrinolyitc type and a hypofibrinolytic type of DIC [30, 31], thus suggesting that the ratio of FDP/DD might be higher in the hyper fibrinolysis group than in the normal fi-

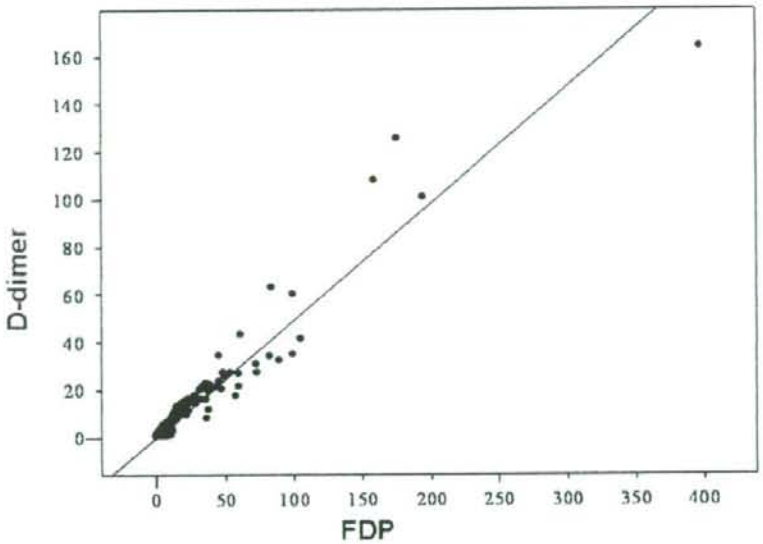

Fig. (3). Relationship between the plasma FDP and plasma D-dimer levels.

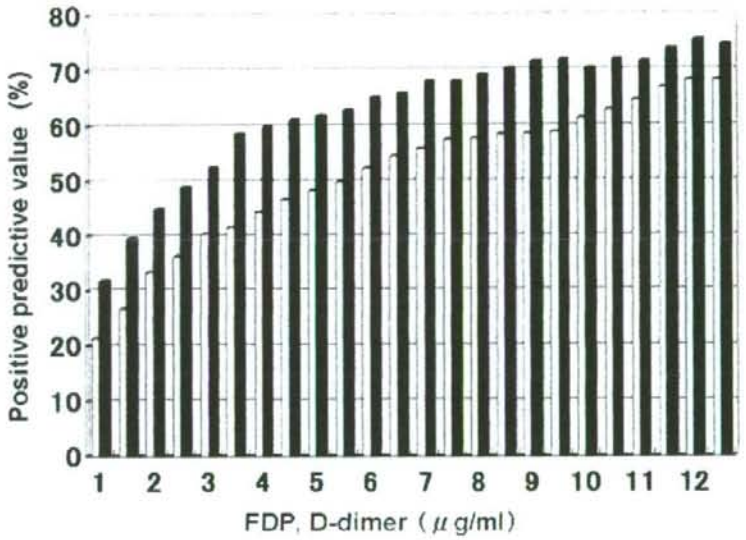

Fig. (4). Positive predictive value for thrombosis according to each FDP or D-dimer level.

brinolysis group. In our study, the number of DIC patients was not sufficient and hyperfibinolysis was not severe. A reduction in the plasminogen activity is also caused by organ failure.

When a D-dimer value of >3.0 μg/ml and a FDP value of >6.0 μg/ml was used, more than 50% of patients had some thrombosis, thus suggesting that these patients need antithrombotic therapy, such as aspirin for atherosclerotic thrombosis or warfarin for venous thrombosis. It is considered that these patients with a high value of FDP or D-dimer were in a hypercoagulable state. D-dimer is useful for the diagnosis of DVT but the cutoff values of D-dimer should be mentioned in each measurement kit. The ROC analysis showed that both FDP and D-dimer were useful for the diagnosis of all types of thrombosis and, in particular, DVT. Since both AUC of D-dimer and FDP were high in the ROC analysis, we believe that both markers are useful for the diagnosis of thrombosis or a hypercoagulable state. The ROC

Table 3. Cutoff Values of Plasma FDP and D-Dimer for DVT/PE

Cutoff value for	FDP		D-Dimer	
	DVT/PE	Thrombosis	DVT/PE	Thrombosis
Cutoff value	7.7 μg/ml	7.6 μg/ml	3.8 μg/ml	3.4 μg/ml
Sensitivity	80.8 %	76.9 %	80.8 %	80.0 %
Specificity	85.3 %	85.2 %	86.1 %	84.7 %
PPV	44.7 %	57.5 %	46.1 %	57.8 %
NPV	96.8 %	93.4 %	96.8 %	94.2 %
Odds ratio	24.42	19.05	26.08	22.16
AUC	0.902	0.875	0.907	0.879

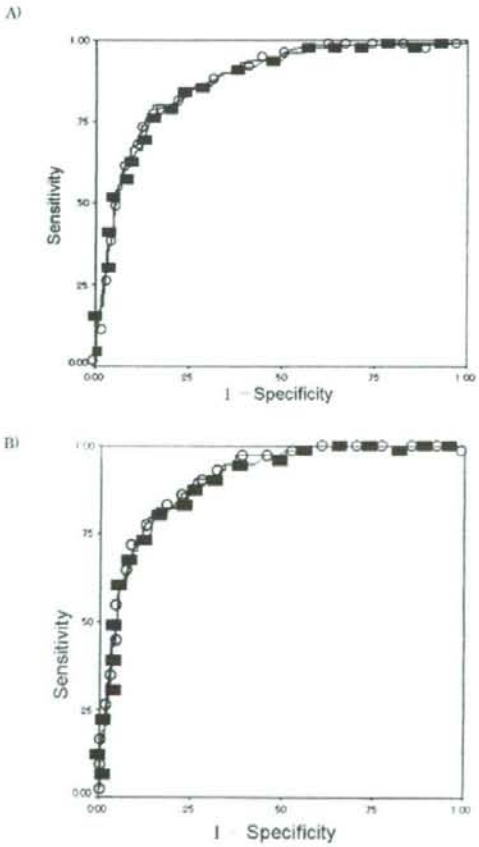

Fig. (5). An ROC analysis for thrombosis or DVT/PE.
A) Thrombosis, B) DVT/PE.

analysis also provided adequate cutoff values of D-dimer and FDP for the diagnosis of all types of thrombosis and DVT/PE. The cutoff values of D-dimer for the diagnosis of all types of thrombosis and DVT/PE were similar, while that of FDP were also similar. Both D-dimer (3.8 μg/ml) and FDP (7.7 μg/ml) had high sensitivity, specificity and NPV but a low positive predictive value. In a previous study [23], soluble fibrin (SF) was reported to be more useful than D-dimer for the diagnosis of thrombosis. The odds ratios of SF for thrombosis, DVT and DIC were markedly high. The cut-off value of soluble fibrin (SF) (7.05 μg/ml) was similar for all types of thrombosis and DVT. A high false positive rate for the D-dimer can potentially result in an increase in pulmonary vascular imaging, an increased length of hospital stay, and increased false positive diagnosis of DVT or PE [32]. Therefore, we strongly consider that the cutoff values of SF and D-dimer for thrombosis should be higher than these values.

In conclusion, our findings suggest that high concentrations of plasma FDP including D-dimer also known as markers for a hypercoagulable state, reflect a high risk for thrombosis. However, a differential diagnosis of various types of thrombosis is difficult if it relies on a fibrin-related marker alone.

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REGULAR ARTICLE

ADAMTS13 related markers and von Willebrand factor in plasma from patients with thrombotic microangiopathy (TMA)

Toshihiko Kobayashi [a], Hideo Wada [b,*], Norihiro Nishioka [a], Masae Yamamoto [a], Takeshi Matsumoto [a], Tomomi Tamaru [a], Shinsuke Nomura [c], Masahiro Masuya [d], Yoshitaka Mori [e], Kaname Nakatani [b], Masakatsu Nishikawa [a], Naoyuki Katayama [a], Tsutomu Nobori [b]

[a] *Department of Hematology, Mie University Graduate School of Medicine, Tsu, Japan*
[b] *Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan*
[c] *Department of Nephrology, Mie University Graduate School of Medicine, Tsu, Japan*
[d] *Department of Blood Transfusion Medicine, Mie University Graduate School of Medicine, Tsu, Japan*
[e] *Mie Red Cross Blood Center, Tsu, Mie, Japan*

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Abstract

The ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type I domain 13) related markers were measured in the plasma of healthy volunteers and thrombotic microangiopathy (TMA) patients including thrombotic thrombocytopenic purpura (TTP) to examine their efficacy in the diagnosis of TTP.
The plasma levels of the ADAMTS13 antigen and ADAMTS13-factor XI complex were significantly lower in TMA patients with a significant decreased ADAMTS13 activity (and these patients were considered to have TTP) than in the healthy volunteers. The plasma levels of ADAMTS13 antigens closely correlated with those of ADAMTS13-factor XI complex. Autoantibody for ADAMTS 13 was also positive in almost all TTP patients. In addition, the ratio of von Willebrand factor (VWF)/ADAMTS13 activity was significantly high in TTP suggesting that this ratio might be more useful for the

* Corresponding author. Tel.: +81 59 232 1111; fax: +81 59 231 5204.
E-mail address: wadahide@clin.medic.mie-u.ac.jp (H. Wada).

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differential diagnosis of TTP than the ADAMTS13 assay alone.
These findings suggest that ADAMTS13 related markers are useful for the diagnosis and analysis of TTP.

Introduction

ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type I domain 13), which was identified in 2001 [1–5], is a zinc metalloprotease, that specifically cleaves the ultra large Von Willebrand factor (VWF) multimer at the Tyr (1605)–Met (1606) bond located in the A2 region of VWF [6,7]. The unusually large Von Willebrand factor (VWF) multimer, produced in and then quickly released from vascular endothelial cells, has often been found in the plasma of patients with familial and non-familial thrombotic thrombocytopenic purpura (TTP) [8,9]. These unusually large VWF multimers have been thought to interact with circulating platelets, thus resulting in platelet clumping due to an elevated shear stress [8]. VWF is a large glycoprotein which is essential for high-shear stress associated platelet adhesion and aggregation [10].

Many studies [6,7,11] have shown low plasma levels of ADAMTS13 activity to be associated with TTP; a life-threatening syndrome characterized by thrombocytopenia and microangiopathic hemolytic

Table 1 Subjects with TMA

ADAMTS13	Age	Sex	Outcome	Activity (%)	Antigen (%)	Activity/ antigen ratio	Complex with FXI (%)	Antibody	VWF (%)	VWF/activity ratio
SL	59	M	Survive	3>	30	NC	27	Positive	272	>90
SL	44	M	Death	3>	108	NC	219	Positive	163	>54
SL	55	M	Survive	3>	63.4	NC	25	Positive	276	>92
SL	34	F	Survive	3>	10	NC	95	Positive	145	>48
SL	46	F	Survive	3>	27.8	NC	41	Positive	199	>65
SL	45	F	Survive	3>	5.9	NC	51	Positive	116	>38
SL	43	F	Survive	3>	15.9	NC	67	Positive	145	>48
SL	45	F	Survive	3>	9.2	NC	13	Negative	47.2	>15
SL	34	F	Survive	3>	90.5	NC	206	Positive	58.3	>19
SL	14	F	Survive	3>	37.6	NC	38	Positive	122	>40
SL	41	M	Death	3>	7.9	NC	31	Positive	145	>48
SL	17	F	Survive	3>	8.2	NC	23	Positive	135	>44
SL	16	F	Survive	3>	7.9	NC	95	Positive	132	>44
SL	61	M	Survive	3>	5.5	NC	18	Positive	153	>51
SL	75	M	Survive	3>	16.6	NC	33	Positive	191	>63
SL	64	F	Survive	3>	57.5	NC	17	Positive	32	>32
SL	79	M	Survive	3>	8.0	NC	13	Positive	178	>59
SL	67	M	Death	12.5	6.2	2.02	26	Positive	188	15.0
SL	50	F	Survive	13.8	8.3	1.66	12	Positive	332	24.2
ML	71	F	Survive	27.5	78.6	0.34	24	Negative	342	12.4
ML	72	F	Death	33.8	25.8	2.13	37	Negative	295	8.7
ML	51	F	Death	38.8	57.9	0.67	23	Negative	222	5.7
ML	38	M	Survive	43.8	55.1	0.79	61	Negative	200	4.6
ML	27	M	Survive	43.8	115	0.38	45	Negative	213	4.9
ML	68	F	Survive	47.5	34.2	1.39	54	Positive	716	15.1
ML	17	M	Survive	48.8	64.5	0.76	74	Positive	38.4	0.8
ML	53	M	Survive	50.0	84.8	0.60	94	Negative	135	2.7
ML	41	M	Death	67.5	81.1	0.83	37	Negative	314	4.7
Normal	28	M	Death	78.8	195	0.4	130	Negative	238	3
Normal	84	F	Survive	80.0	61.4	1.93	96	Negative	312	3.9
Normal	24	M	Survive	96.3	175	0.55	154	Negative	180	1.9
Normal	44	F	Death	98.8	106	0.93	44	Negative	228	2.3
Normal	51	F	Survive	128.8	168	0.76	97	Negative	202	1.6

SL; significantly low, ML; moderate low, NC; not calculated.

Table 2 Familial TMA

Family	Age	TMA	Sex	Activity (%)	Antigen (%)	Activity/antigen ratio	Complex with FXI (%)	Antibody	VWF (%)	VWF/activity ratio
1	21	+	F	143.8	200	0.72	88	Negative	102	0.7
2	1	+	M	112.5	102	1.1	64	Negative	54.8	0.5
2	24	+	F	121.3	120	1	77	Negative	57.1	0.5
2	26	+	M	82.5	119	0.69	59	Negative	72.7	0.9
3	54	+	F	118.8	62.2	1.9	103	Negative	108	0.9
3	30	+	F	200	71.7	2.79	97	Negative	185	0.9
4	73	−	M	36.3	182	0.20	84	Negative	77.2	2.1
4	44	+	M	3>	4.1	NC	12	Negative	227	>79
4	69	−	F	37.1	155	0.24	29	Negative	58.5	1.60

SL; significantly low, ML; moderate low, NC; not calculated.

anemia, which is often associated with neurological dysfunction, renal failure, and fever [12,13]. Thrombotic microangiopathy (TMA) including TTP is often observed after bone marrow transplantation [14] or liver transplantation [15].

A severely deficient ADAMTS13 activity (less than 5% of that in normal plasma) is caused by either a mutation of the ADAMTS13 gene [2,16] or by inhibitory antibodies against ADAMTS13 [17–19]. Since measuring the ADAMTS13 activity is important in the diagnosis of TTP, Kokame developed a fluorescence resonance energy transfer (FRET) assay to determine the ADAMTS13 activity [20]. An assay of an inhibitor for ADAMTS13 which measures of the activity requires a high level of skill and quite a long time to diagnose acquired TTP. There are several patients with TTP who possess normal levels of ADAMTS13 activity [19], thus suggesting that other biological factors in addition to ADAMTS13 may therefore play a role in TTP. A recent report demonstrated that ADAMTSA13 is able to form a stable complex with FXI and FXIa and these complexes are found in plasma [21].

In this study, the ADAMTS13 related markers were measured in the plasma of 50 healthy volunteers and 40 patients with TMA and thus examined the usefulness of a diagnosis of TMA, especially TTP.

Materials and methods

The activity and antigen of ADAMTS13, ADAMTS13–FXI complex and VWD antigen were measured in 50 healthy volunteers (19 females and 31 males; median age, 31 years; range, 19–51 years) and in 40 patients with TMA (22 females and 18 males; median age, 44 years; range, 1–84 years) (Table 1). In the 40 patients with TMA, who were admitted to either Mie University Hospital or the affiliated hospital from 1996 to 2006, 4 families including 7 patients demonstrated congenital TMA (Table 2). Regarding the underlying diseases, there were 15 autoimmune diseases, but there was no post bone marrow transplantation, post liver transplantation nor patients with verotoxin producing *Escherichia coli* infection. The diagnosis of TMA was made based on the presence of thrombocytopenia due to the consumption, microangiopathic hemolytic anemia, neurological abnormalities, renal function impairment and high fever [19]. In TMA, the patients who had less than 20% of ADAMTS13 are considered to have TTP.

Although these patients were treated with plasma exchange, plasma transfusion, anti-platelet drug or steroid, 8 patients with TMA died.

The study protocol was approved by the Human Ethics Review Committee of Mie University School of Medicine and a signed consent form was obtained from each subject.

Blood sampling was done in on admission or in the diagnosis of TMA. Human plasma was obtained by centrifugation at 3000 g at 4 °C for 15 min from whole blood that was treated with a 1/10 volume of 3.8% sodium citrate as an anti-coagulant.

The ADAMTS13 activity was measured by a fluorescent assay and the fluorogenic substrate, FRETS-VWF73, was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) [20]. The assay was performed according to the method of Kokame et al. [20]. In healthy volunteers, the ADAMTS13 activity (median value; 25% tile–75% tile) (113.0%; 61.1%–261.5%) was not being normally distributed (Table 3). ADAMTS13 antigen, ADAMTS13–FXI complex and the inhibitor of ADAMTS13 were measured by ELISA using an IMUBIND®ADAMTS13 ELISA (American Diagnostica Inc.; ADI, CT, USA), IMUBIND® ADAMTS13/FXI Complex ELISA (ADI) and IMUBIND®ADAMTS13 Autoantibody ELISA (ADI), respectively. In healthy volunteers, ADAMTS13 antigen (95.4%; 60.4%–177.0%) and ADAMTS13–FXI complex (101.0%; 37.0%–264.0%) were not being distributed normally (Table 3). In an assay of autoantibody for ADAMTS13, the 95% CI of the healthy volunteers ranged from 2.75 to 19.55 AU/ml, thus suggesting that more than 20 AU/ml of autoantibody is considered to be positive for inhibitor. The VWD antigen was measured using a VIDAS VWF (BIOMÍRIEUX, Marcy l'Etoile, France). In healthy volunteers, VWF antigen (113%; 61.1%–261.5%) were not being normally distributed, and VWF antigen/ADAMTS13 activity ratio was 1.05; 0.55–3.64 (Table 3).

Table 3 ADAMTS13 related markers in healthy volunteers

	Median	95% CI
VWF antigen (%)	113	61.1–261.5
ADAMTS13 activity (%)	106.6	65.8–153.5
ADAMTS13 antigen (%)	95.4	60.4–177.0
ADAMTS13–FXI complex (%)	101.0	37.0–264.0
Autoantibody (AU/ml)	7.8	2.8–19.5
ADAMTS13 activity/antigen ratio	1.11	0.55–3.64
VWF antigen/ADAMTS13 activity ratio	1.05	0.55–3.64

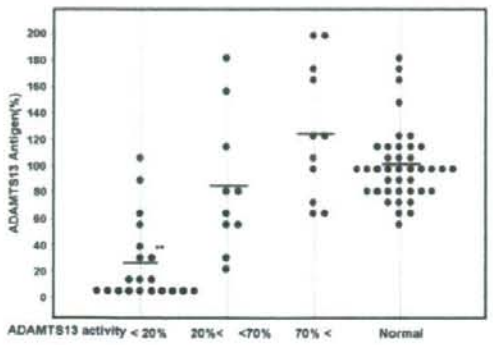

Figure 1 Plasma levels of ADAMTS13 antigen in patients with TMA.

Statistical analysis

The data are expressed as the mean ± SD. The differences between the groups were examined for statistical significance using the Mann–Whitney's *U* test. The correlation between the groups was examined for statistical significance using Pearson's correlation analysis. A *p* value of less than 0.05 was considered to indicate the presence of a statistically significant difference.

Results

The plasma levels of VWF antigen (median value; 25% tile–75% tile), were significantly higher in patients with TMA (180.0%; 29.4% –538.4%) than in the healthy volunteers ($p<0.01$). While the plasma levels of ADAMTS13 antigen (20.6%; 0%–171.9%) and ADAMTS13–FXI complex (32.0%; 8.0%–212.0%) were significantly lower in those with TMA than in the healthy volunteers ($p<0.01$). The ADAMTS13 activity of 20 patients with TMA (19 acquired and 1 familial patients) was less than 20% (significant low group) and that of 10 patients with TMA was from 20% to 70% (moderate low group) and that of 11 patients with TMA was higher than 70% (normal group). The plasma levels of ADAMTS13 antigen were also significantly lower in the patients with significant low ADMTS13 activity (9.6%; 7.9%–33.8%) than those with moderate

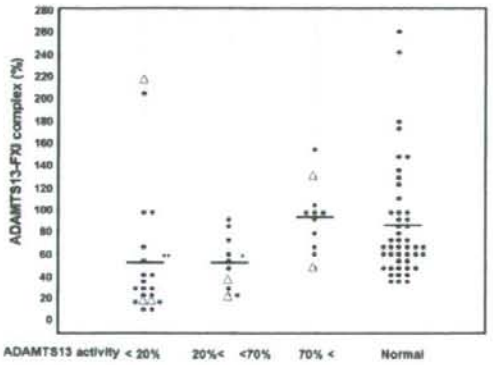

Figure 2 Plasma levels of ADAMTS13–FXI complex in patients with TMA △; patients who died during clinical course of TMA.

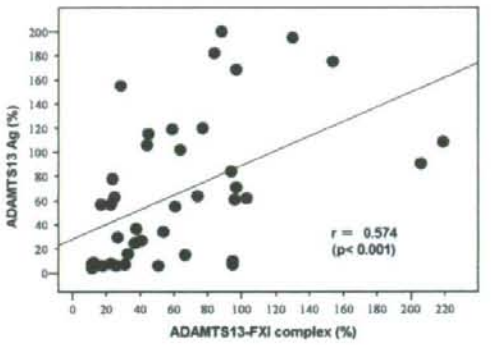

Figure 3 Relationship between ADAMTS13 antigen and ADAMTS13–FXI complex in patients with TMA. $Y=28.724+0.606X$, $r=0.574$, $p<0.001$.

low (71.6%; 55.1%–115.0%) or with normal (119.0%; 79.3%–173.3%) ADAMTS13 activity, and healthy volunteers ($p<0.01$, respectively) (Fig. 1). In patients with less than 3% of ADAMTS13 activity, ADAMTS13 antigen was able to be detected and ADAMTS13 antigen was not well correlated with ADAMTS13 activity. Autoantibody for ADAMTS13 was positive in 18/19 of significantly low ADAMTS13 group and in 2/9 of moderate low ADAMTS13 group but negative in all of normal ADAMTS13 group and familial TMA group, showing that the results from autoantibody ELISA were similar to those from the inhibitor assay by FRETS-VWF73.

The plasma levels of ADAMTS13–FXI complex were widely distributed and significantly lower in the patients with significant low AAMTS13 activity (29.0%; 17.5%–59.0%) than in normal ADAMTS13 group (96.0%; 67.3%–101.5%) and healthy volunteers ($p<0.001$, respectively, Fig. 2). Two patients with low ADAMTS 13 activity had high ADAMTS13–FXI complex and high ADAMTS13 antigen. The plasma levels of ADAMTS13–FXI complex also tend to be low in the patients who died during the clinical course of TMA. The plasma levels of ADAMTS13–FXI complex were also correlated with ADAMTS 13 antigen ($Y=28.724+0.606X$, $r=0.574$, $p<0.001$, Fig. 3).

Although there was no significant difference in the VWF antigen levels among the significant low (149%; 127%–195%),

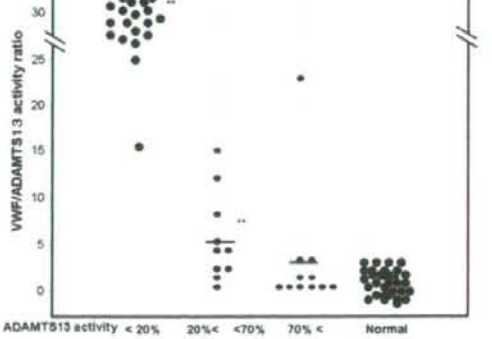

Figure 4 Ratio of VWF/ADAMTS13 activity in patients with TMA.

moderate low (206%; 77%–295%) and normal ADAMTS 13 (180%; 80%–222%) groups, the ratio of VWF/ADAMTS13 activity was significantly higher in the patients with TMA than in the healthy volunteers ($p<0.001$). The ratio of VWF/ADAMTS 13 activity was significantly higher in the significantly low ADAMTS 13 group than the moderate low (4.7; 2.1–8.7) and normal ADAMTS13 (0.9; 0.7–2.7) group (Fig. 4). The cutoff levels of VWF/ADAMTS 13 activity ratio between TTP and other TMA appear to be 15.0. The patients with familial TTP had no detectable inhibitor, and only one family showed a low ADAMTS13 activity and a high VWF/ADAMTS13 ratio (Table 3).

Discussion

The plasma levels of the VWF antigen were significantly high in patients with TMA. VWF is released from vascular endothelial cells as is thrombomodulin and plasminogen activator inhibitor 1 (PAI-I) and they are referred to as injured vascular endothelial cell injured markers [22]. These markers are significantly elevated in patients with a poor outcome [23], thus suggesting that these markers reflect the outcome. Furthermore, the ratio of VWF/ADAMTS13 activity was significantly higher in TMA, thus suggesting that the ultra large multimers of VWF accelerate microthrombus formation by activated platelets. According to our findings, the ratio might therefore be more useful for the diagnosis of TTP than ADAMTS13 alone.

ADAMTS13 was recently identified to be a new hemostatic factor, which was previously called VWF cleaving protease [24]. Either congenital or acquired defects of the enzymatic activity of this protein lead to TTP [19,25]. ADAMTS13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF which defines the minimal region that can be recognized as a specific substrate by ADAMTS13 [26].

More than half of TMA patients who have significantly low ADAMTS 13 activity are considered to have TTP and these patients also had significant low ADAMTS13 antigen levels. The activity of ADAMTS13 did not show a close correlation with the presence of the ADAMTS13 antigen. Even in patients with less than 3% of ADAMTS13 activity, they had detectable levels of ADAMTS13 and the activity/antigen ratio of ADAMTS13 was also significantly low in patients with TMA. These findings suggest that the inhibitor not only induces the clearance of ADAMTS13 protein in the circulation but it also inhibits the activity of ADAMTS13. In an assay for the inhibitor of ADAMTS13, 18 of 19 TMA patients with significant low ADAMTS13 activity were positive for the autoantibody. These findings show that the results from autoantibody ELISA were similar to those from the inhibitor assay using FRETS-VWF73.

The plasma levels of ADAMTS13–FXI complex tend to be widely distributed and significantly low in TMA patients with significant low ADAMTS13 activity. This presence of this complex is well correlated to the presence of the ADAMTS 13 antigen. ADAMTSA13 forms a stable complex with FXI and FXIa and these complexes are found in the plasma [21]. Although the role of ADAMTS13–FXI complex remains unclear, the measurement of ADAMTS13–FXI complex and ADAMTS 13 antigen may be useful for the diagnosis of TTP.

The ADAMTS13 activity was significantly low in both the patients with TTP and in those belonging to the TTP family, thus indicating that ADAMTS13 plays an important role in the onset of TTP. However, 6 patients had an ADAMTS13 activity of more than 60%, thus suggesting that the TTP in these patients may have been caused by abnormalities in other factor such as Factor H [27] and CD46 [28].

In 40 patients with TMA, 8 patients died within 3 months, while 32 patients had a complete remission. There was no significant difference in the VWF antigen, ADAMTS13 activity, ADAMTS13 antigen, ADAMTS13–FXI complex, inhibitor, activity/antigen ratio of ADAMTS13 or the ratio of VWF/ADAMTS13 activity between survivors and non-survivors. Then, the predictive marker for the outcome of TTP may be required.

In summary, the measurement of the ADAMTS13 activity, as well as the presence of ADAMTS13 antigen and the complex associated with the FXI of ADAMTS13 might therefore be useful for the diagnosis of TTP.

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Regular article

Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis ☆

Toshiyuki Miyata [a,*], Yukiko Sato [a], Junko Ishikawa [a], Hiromi Okada [a], Satoshi Takeshita [b], Toshiyuki Sakata [c], Koichi Kokame [a], Rina Kimura [a], Shigenori Honda [a], Tomio Kawasaki [d], Etsuji Suehisa [e], Hajime Tsuji [f], Seiji Madoiwa [g], Yoichi Sakata [g], Tetsuhito Kojima [h], Mitsuru Murata [i], Yasuo Ikeda [j]

[a] National Cardiovascular Center Research Institute, Suita, Japan
[b] Department of Medicine, National Cardiovascular Center, Suita, Japan
[c] Laboratory of Clinical Chemistry, National Cardiovascular Center, Suita, Japan
[d] Cardiovascular and Thoracic Surgery, Osaka University Graduate School of Medicine, Suita, Japan
[e] Laboratory for Clinical Investigation, Osaka University Hospital, Suita, Japan
[f] Division of Blood Transfusion and Cell Therapy, Kyoto Prefectural University of Medicine, Kyoto, Japan
[g] Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan
[h] Department of Medical Technology, Nagoya University of School of Health Sciences, Nagoya, Japan
[i] Department of Laboratory Medicine, Keio University, Tokyo, Japan
[j] Department of Internal Medicine, Keio University, Tokyo, Japan

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ABSTRACT

Introduction: Genetic deficiencies of *PROS1*, *PROC*, and *SERPINC1* (antithrombin) are risk factors for deep vein thrombosis (DVT). Diagnosis of the inherited deficiencies of these three genes is sometimes difficult because of the phenotypic variability. This study was undertaken to reveal the frequency of nonsynonymous mutations of these three genes in Japanese DVT patients.
Patients/Methods: One hundred seventy-three DVT patients were registered by the Sub-group of Blood Coagulation Abnormality, from the Study Group of Research on Measures for Intractable Diseases. We sequenced the entire coding regions of the three genes in all DNA samples and identified the nonsynonymous mutations.
Results and Conclusions: For *PROS1* we identified 15 nonsynonymous mutations in 28 DVT patients; for *PROC*, 10 nonsynonymous mutations in 17 patients; and for *SERPINC1*, 13 nonsynonymous mutations in 14 patients. Five patients had two mutations in *PROS1* and *PROC*, and all of them had *PROS1* K196E mutation. We previously identified one patient with a large *PROS1* gene deletion. Thus, 55 out of 173 patients (32%) carried at least one genetic defect in the three genes. The *PROS1* K196E mutation found in 15 Japanese DVT patients was the most prevalent. Mutations of *PROC* K193del and V339M were the second, each found in four patients. Our data suggested that the *PROC* K193del mutation caused the loss of the anticoagulant activity but not the amidolytic activity. Our effort is the first DNA resequencing study to identify the genetic variations in DVT patients without any consideration of their plasma activities and antigens. To minimize selection bias in a future evaluation of the contribution of genetic deficiency to DVT, we must recruit patients consecutively.

Introduction

Genetic deficiencies of protein S, protein C, and antithrombin (*PROS1*, *PROC*, *SERPINC1*) are well known risk factors for deep vein thrombosis (DVT) [1–4]. These rare deficiencies are present in less than 10% of Caucasian patients with DVT [5]. In the Caucasian population, two common genetic mutations, factor V Leiden and prothrombin G20210A, have been recognized as additional causes of DVT [6]. These two common genetic mutations have extreme difference in prevalence among various ethnic groups and are absent in the Japanese population [7]. There is growing evidence that within different ethnic groups, mutations associated with disease arise with different frequencies [8]. Recently, it was found that protein S mutation K196E is a genetic risk factor for DVT in the Japanese population [9,10]. We estimated the allele frequency to be 0.009 based on the observation that 66 out of 3,651 Japanese in the general population carried the mutant E allele [9] and that heterozygous carriers showed 16% lower plasma protein S activity than wild-type individuals [11].

Abbreviations: DVT, deep vein thrombosis; PCR, polymerase chain reaction.

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* Corresponding author. National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012x2512; fax: +81 6 6835 1176.
E-mail address: miyata@ri.ncvc.go.jp (T. Miyata).

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The prevalence of inherited genetic deficiencies for DVT has been intensively studied so far. In unselected Caucasian patients with DVT, rare mutations in *PROS1*, *PROC*, and *SERPINC1* were observed in about 8% of patients; in selected DVT patients, these mutations were observed in about 13% [6,12]. When two common mutations, factor V Leiden and prothrombin G20210A, were included, the prevalence was increased to more than 30% of unselected DVT patients and in 41–69% of selected DVT patients [6,12]. Most of the genetic analyses reported so far have been examined in DVT patients with low plasma activity, and no sequencing efforts for DVT patients without consideration of their activity and antigen levels have been undertaken. Thus, the prevalence of inherited mutations in *PROS1*, *PROC*, and *SERPINC1* in patients with DVT might be underestimated.

To identify genetic risk factors for DVT in Japanese, we previously enrolled 161 Japanese DVT patients. We genotyped five functional genetic variations in patients with DVT and in the general population and identified protein S mutation K196E as a genetic risk factor [9]. In the present study, we sequenced the entire coding regions of *PROS1*, *PROC*, and *SERPINC1* in DNA samples of the previously enrolled 159 Japanese DVT patients and additional 14 DVT patients without consideration of their plasma activities and antigens. As a result, we identified various nonsynonymous mutations in the three genes. We had previously identified one patient with a large *PROS1* gene deletion [13]. In total, 55 out of the 173 enrolled patients (32%) carried at least one mutation in either *PROS1*, *PROC*, or *SERPINC1*. This is the first DNA sequencing study to identify the genetic variations in DVT patients without any consideration of their plasma activities and antigens.

Materials and Methods

Patients

One hundred and seventy-three DVT patients were enrolled by the Study Group of Research on Measures for Intractable Diseases working under the auspices of the Ministry of Health, Labor and Welfare of Japan, as described previously [9]. The patients consisted of the previously enrolled 159 Japanese DVT patients and additional 14 DVT patients. Diagnosis of DVT was made by ultrasonography, radioisotope venography, and magnetic resonant imaging angiography. Patients had a mean age of 46.6 years (range 22-87). In 16% of patients, thrombosis was recurrent, and in 13%, thrombosis had occurred in family members. The protocol of this study has been approved by the Ethical Review Committee of each institute.

Only those who gave written informed consent for genetic analyses were included in this study.

Direct DNA sequencing of PROS1, PROC, and SERPINC1

We sequenced all exons, flanking regions, and promoter regions of *PROS1*, *PROC*, and *SERPINC1* in 173 DVT patients [14]. The method of direct sequencing using the 96-capillary 3730xl DNA Analyzer (Applied Biosystems Japan, Tokyo, Japan) has been described previously [15,16]. Information on the primers and polymerase chain reaction (PCR) conditions is available on request. The obtained sequences were examined for the presence of mutations using NAMIHEI (version 1.0; Mitsui Knowledge Industry, Tokyo, Japan) and Sequencher software (version 4.2.2; Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [17]. We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide +1, and the initial Met residue is denoted amino acid +1 [18].

Activity measurements in patients with nonsynonymous mutations

Protein S anticoagulant activity was measured as cofactor activity for activated protein C on the basis of the activated partial thromboplastin time assay. Protein C amidolytic activity was measured using the chromogenic substrate and Protac derived from *Agkistrodon contortrix* as the activator. Antithrombin activity was measured as a heparin cofactor activity using the chromogenic substrate. The assay has been performed at each institution.

Statistical analysis

Comparisons between mutation carriers and non-carriers were analyzed by t-test. Differences with P values less than 0.05 were considered statistically significant.

Results

Nonsynonymous mutations in PROS1

We sequenced all exons, flanking regions, and approximately 200 bp of the promoter region in *PROS1*, and identified 15 nonsynonymous mutations in 173 Japanese patients with DVT (Table 1). These mutations included 10 missense mutations, 3 splice-site mutations, and 2 frameshift mutations. The K196E mutation was prevalent with

Table 1
List of nonsynonymous mutations identified in the *PROS1* gene in 173 Japanese DVT patients

Relative position from ATG		Nuc.	Region/ Domain	Amino acid change	Major homo	Hetero	Minor homo	Total	Minor allele freq.	5' Near Seq. 20 bp	3' Near Seq. 20 bp
Genomic DNA	cDNA										
46342	77-1	G/C	Int1	Splicing	172	1	0	173	0.003	taaactgattgtttccttca	ttttgtcaaagcaacaggct
49510	259	G/C	Ex3/TSR	Val87Leu	171	2	0	173	0.006	attttatccaaaatactta	gtaagttcaaaacatctcaa
63044	260-1	G/A	Int3	Splicing	172	1	0	173	0.003	tatgtttgtttttaattttca	tttgtcttcgctcttttttcaa
63086	301	C/T	Ex4/TSR	Arg101Cys	172	1	0	173	0.003	ctgggttattcactgctgca	gtcagtcaactaatgcttat
67676	416	C/T	Ex5/EGF1	Ala139Val	172	1	0	173	0.003	gagctgcaaagatggaaaag	ttcttttacttgcacttgta
67951	586	A/G	Ex6/EGF2	Lys196Glu	158	13	2	173	0.049	gtttgtatgctttcaaat	agaaagattgtaaaggtaag
75209	756	T/G	Ex8/EGF4	Cys252Trp	172	1	0	173	0.003	gaatgctctgagaacatgtg	gctcagcttttgttgtcaatta
80726	1064	G/A	Ex10/SHBG	Arg355His	172	1	0	173	0.003	gtggctcctgattgcacttc	tggtggaaagattgaagttc
80732	1070	G/T	Ex10/SHBG	Gly357Val	172	1	0	173	0.003	cctgattgcacttcgtggtg	aaagattgaagttcagctta
80763	1101	1/2*	Ex10/SHBG	Thr368TyrfsX9	172	1	0	173	0.003	gttcagcttaagaatgaaca	acatccaaaatcacaactgg
80810_80811	1148_1149	1/2**	Ex10/SHBG	Asn384GlyfsX9	172	1	0	173	0.003	tgttattaataatggtctat	aatatggtacgtttgcagat
87338	1247	C/A	Ex11/SHBG	Pro416Gln	172	1	0	173	0.003	acctggacccctttttaagc	ggaaaatggattgctggaaa
89016	1486	G/T	Ex12/SHBG	Asp496Tyr	172	1	0	173	0.003	gaattgctcaatttcacata	attatagtaagtgattttcc
96772	1858	G/A	Ex14/SHBG	Gly620Ser	172	1	0	173	0.003	caaaagtggccacatacctg	gtggccttccaggtatctgc
96785	1870+1	G/A	Int14	Splicing	172	1	0	173	0.003	atacctgggtggccttccag	tatctgcttactttttcttc

*1: T, 2: TT, T insertion, **1: (GG)1, 2: (GG)2, GG insertion.
The A of the ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1.
Int: intron, Ex: exon, TSR: thrombin sensitive region, EGF: epidermal growth factor like domain, SHBG: sex hormone binding globulin region.
Thr368TyrfsX9: Frameshift mutation at Thr368 to Tyr resulting in the stop codon after 9 amino acids. Asn384GlyfsX9: Frameshift mutation at Asn384 to Gly resulting in the stop codon after 9 amino acids.
The nucleotide sequence (GenBank Accession ID: NC_000003.9) was used as a reference sequence.

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Table 2
List of nonsynonymous mutations identified in the *PROC* gene in 173 Japanese DVT patients

Relative position from ATG		Nuc.	Region/ Domain	Amino acid change	Major homo	Hetero	Minor homo	Total	Minor allele freq.	5' Near Seq. 20 bp	3' Near Seq. 20 bp
Genomic DNA	cDNA										
1469	199	G/A	Ex3/Gla	Glu67Lys	172	1	0	173	0.003	tagaggagatctgtgacttc	aggaggccaaggaaatttic
3229	400	G/T	Ex5/EGF1	Glu134stop	172	1	0	173	0.003	agggccgcttctgccagcgc	gtgaggggagaggtggatg
3377	446	A/C	Ex6/EGF2	His149Pro	172	1	0	173	0.003	ggacaacggcggctgcacgc	ttactgcctagaggaggtgg
6184_6186	577_579	AAG/–	Ex7/LC tail	Lys193del	169	4	0	173	0.012	cctggaagcggatggagaag	cgcagtcacctgaaacgaga
6188	581	G/A	Ex7/LC tail	Arg194His	172	1	0	173	0.003	gaagcggatggagaagaagc	cagtcacctgaaacgagaca
6238	631	C/T	Ex7/AP	Arg211Trp	171	2	0	173	0.006	aagaagaccaagtagatccg	ggctcattgatgggaagatg
6266	659	G/A	Ex7/Cat	Arg220Gln	172	1	0	173	0.003	tgatgggaagatgaccaggc	gggagacagccccttggcagg
8429	811	C/T	Ex9/Cat	Arg271Trp	172	1	0	173	0.003	ctgcaggagagtatgacctg	gccgctgggagaagtgggag
8633	1015	G/A	Ex9/Cat	Val339Met	169	4	0	173	0.012	aggccggccaggagaccctc	tgacgggctgggggctaccac
8661	1043	G/A	Ex9/Cat	Arg348Gln	172	1	0	173	0.003	ctgggctaccacagcagcc	agagaaggaggccaagagaa

The A of the ATG of the initiator Met codon is denoted nucleotide + 1, and the initial Met residue is denoted amino acid + 1.
Ex: exon, Gla: Gla domain, EGF: epidermal growth factor like domain, LC tail: C-terminal portion in the light chain, AP: activation peptide, Cat: catalytic domain.

an allele frequency of 0.049 including 2 homozygotes and 13 heterozygotes, as has already been reported [9]. Two patients had the V87L mutation. Thirteen other nonsynonymous mutations were found in each of 13 patients. One patient had two mutations, R101C and K196E. To determine whether the two mutations were located on the same allele, PCR products including the two mutations were subcloned into a plasmid and 9 independent clones were sequenced. Each clone contained one mutation, indicating that the patient was a compound heterozygote with R101C and K196E mutations. Another patient had P416Q and D496Y mutations. We sequenced subcloned PCR products and found that the two mutations were on the same allele. We had previously examined whether the large *PROS1* gene deletion was present in the DVT patients and found one patient with the large *PROS1* gene deletion [13]. In all, 29 patients accounting for 17% of the 173 patients carried one or two nonsynonymous mutations or the large gene deletion in the *PROS1* gene and 15 patients accounting for 9% had the K196E mutation.

Nonsynonymous mutations in PROC

We sequenced all exons and flanking regions in *PROC*. The initial ATG codon of *PROC* is located in exon 2. We sequenced approximately 330 bp of the upstream of exon 1 as the putative promoter regions. We identified 10 nonsynonymous mutations, including 8 missense mutations, 1 nonsense mutation, and 1 in-frame deletion (Table 2). Three nonsynonymous mutations were found in more than one patient: K193del in 4 patients, R211W in 2 patients, and V339M in 4 patients. In all, 17 patients accounting for 10% of our 173 patients carried nonsynonymous mutations of the *PROC* gene.

Nonsynonymous mutations in SERPINC1

We sequenced all exons, flanking regions, and approximately 1.0 kb of the promoter region in *SERPINC1*. We identified 13 nonsynonymous mutations, including 8 missense mutations, 2 frameshift mutations, 2 nonsense mutations, and 1 splice–site mutation (Table 3). One frameshift mutation, L256fsX9, was found in 2 patients. In all, 14 patients accounting for 8% of our 173 patients carried nonsynonymous mutations of the *SERPINC1* gene.

Activity of protein S, protein C, and antithrombin in patients with nonsynonymous mutations

We have measured plasma activities of protein S, protein C, and antithrombin in patients with nonsynonymous mutations (Table 4). Heterozygous patients with the *PROS1* K196E mutation showed low protein S anticoagulant activity and homozygote of this mutation showed even lower activity. We measured the protein C amidolytic activity in three patients with the *PROC* K193del mutation and found that all showed more than 90% activity. Previous studies reported that the protein C amidolytic activity in patients with this mutation was normal but the anticoagulant activity was low [10,19]. Three patients with the *PROC* V339M mutation showed low protein C amidolytic activity but one patient showed the normal level (Table 4).

Table 3
List of nonsynonymous mutations identified in the *SERPINC1* gene in 173 Japanese DVT patients

Relative position from ATG		Nuc.	Region	Amino acid change	Major homo	Hetero	Minor homo	Total	Minor allele freq.	5' Near Seq. 20 bp	3' Near Seq. 20 bp
Genomic DNA	cDNA										
2346_2348	47_49	3/2*	Ex2	Tyr17IlefsX14	172	1	0	173	0.003	ttgtctccttgcaggaagg	atcttttgtcctgctgctc
5317	480	C/G	Ex3	Cys160Trp	172	1	0	173	0.003	ttctttgccaaactgaactg	cgactctatcgaaaagccaa
6429	685	C/T	Ex4	Arg229stop	172	1	0	173	0.003	tgtccaataagaccgaaggc	gaatcaccgatgtcattccc
6481	737	T/A	Ex4	Val246Glu	172	1	0	173	0.003	caatgagctcactgttctgg	gctggttaacaccatttact
6506	762	G/C	Ex4	Lys254Asn	172	1	0	173	0.003	gttaacaccatttactttcaa	gtactcagaatggccctgga
7322	767	1/2*	Ex5	Leu256fsX9	171	2	0	173	0.006	tgtgattctcttccaggtcc	gtggaagtcaaagttcagcc
7368	813	C/G	Ex5	Phe271Leu	172	1	0	173	0.003	aacacaaggaaggaactgtt	tacaaggctgatggagagtc
7431	876	T/A	Ex5	Tyr292stop	172	1	0	173	0.003	caggaaggcaagttccgtta	cggcgcgtggctgaaggcac
7435	880	C/T	Ex5	Arg294Cys	172	1	0	173	0.003	aaggcaagttccgttatcgg	gcgtggctgaaggcacccag
9745	1154-1	G/A	Int5	Splicing	172	1	0	173	0.003	cttctccggtcttcctttcca	gtattgttgcagaaggcrga
9791	1199	T/C	Ex6	Phe400Ser	172	1	0	173	0.003	cctctatgtctcagatgcat	ccatagggcatttcttgagg
13253	1277	C/T	Ex7	Ser426Leu	172	1	0	173	0.003	tgttgtgattgctggccgtt	gctaaaccccaacagggtga
13291	1315	C/A	Ex7	Pro439Thr	172	1	0	173	0.003	tgactttcaaggccaacagg	ctttcctggttttttataaga

*3: TTT, 2: TT, T deletion, Int: intron, Ex: exon.
The A of the ATG of the initiator Met codon is denoted nucleotide + 1, and the initial Met residue is denoted amino acid + 1.
Thr17IlefsX14: Frameshift mutation at Thr17 to Ile resulting in the stop codon after 14 amino acids. Leu256fsX9: Frameshift mutation at Leu256 resulting in the stop codon after 9 amino acids.
The nucleotide sequence (GenBank Accession ID: NC_000001.9) was used as a reference sequence.

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Table 4
Activity levels in patients with nonsynonymous mutations *PROS1* mutation

PROS1 mutation	anticoagulant activity, %
Splicing, intron 3	<10
Ala139Val	28
Lys196Glu, hetero*	60, 49
Lys196Glu, homo	39
Gly357Val	31
Pro416Gln/Asp496Tyr**	26
Gly620Ser	<10
Splicing, intron 14	18
PROC mutation	amidolytic activity, %
Glu67Lys	70
Glu134stop	54
His149Pro	44
Lys193del***	90, 112, 130
Arg194His	77
Arg211Trp	35
Arg220Gln	64
Arg271Trp	77
Val339Met****	28, 40, 38, 77
Arg348Gln	127
SERPINC1 mutation	activity, %
Tyr17IlefsX14	44
Cys160Trp	51
Arg229stop	52
Val246Glu	53
Lys254Asn	50
Leu256fsX9*	52, 52
Phe271Leu	56
Tyr292stop	48
Arg294Cys	96
Splicing, intron 5	50
Phe400Ser	53
Ser426Leu	113
Pro439Thr	58

*Two patients, **Pro416Gln and Asp496Tyr were on the different alleles, ***Three patients, ****Four patients.

Patients with mutations in two genes

Five patients had nonsynonymous mutations in both *PROS1* and *PROC* (Table 5). All had the *PROS1* K196E mutation. As described, two patients had two different mutations in the *PROS1* gene and one patient had the large *PROS1* gene deletion [13]. Thus, as the results of extensive sequencing of the three genes in 173 DVT patients, 24 patients carried nonsynonymous mutations only in *PROS1*, 12 carried nonsynonymous mutations only in *PROC*, and 14 carried nonsynonymous mutations only in *SERPINC1* (Table 6). Thus, in total, 55 patients accounting for 32% of the 173 patients carried one or two nonsynonymous mutations in three genes (Table 6).

Comparison of the first onset age of DVT between mutation carriers and non-carriers

We compared the first onset age of DVT between mutation carriers and non-carriers. The onset age of mutation carriers (n=55: 44.7 +/- 16.5 years old, mean +/- SD) was significantly earlier than that of non-carriers (n=118, 52.6 +/- 16.1 years old, p=0.0031) (Table 7). Family history of DVT in mutation carriers (12 out of 41, percent of family history: 29%) was significantly higher than that in non-carriers (8 out of 93, percent of family history: 9%, p=0.0034).

Table 5
Five patients with nonsynonymous mutations in two genes

PROS1	PROC	DVT onset age, year	Family history
Lys196Glu	Lys193del	57	unavailable
Lys196Glu	Arg221Trp	40	no
Lys196Glu	Arg271Trp	39	yes
Lys196Glu	Val339Met	25	yes
Lys196Glu	Val339Met	55	no

Table 6
Number of patients with nonsynonymous mutations

mutated gene	number of patients
PROS1	24*
PROC	12
SERPINC1	14
PROS1+PROC	5**
Total	55

*Ten patients had Lys196Glu mutation and one patient had a large gene deletion. ** Five patients had *PROS1* Lys196Glu mutation.

Discussion

We sequenced *PROS1*, *PROC*, and *SERPINC1* genes in DNA samples from 173 Japanese DVT patients and found that 54 patients carried 38 nonsynonymous mutations such as missense, frameshift, splice-site, in-frame, and nonsense mutations. We had already identified one patient with the large *PROS1* gene deletion among our DVT patients [13]. Thus, in our DVT population, about 32% patients (55 out of 173) carried nonsynonymous mutations or the large gene deletion in at least one of the above three genes. This is the first DNA sequencing study to identify the genetic mutations in DVT patients without any consideration of their plasma activities and antigens.

We have previously measured plasma activities of protein S, protein C, antithrombin, and plasminogen in a general population consisting of about 4,500 individuals [20–22] and found that the standard deviations of antithrombin and plasminogen were relatively small (12.3% and 15.9%, respectively) whereas those of protein S and protein C were large (20.8% and 21.1%, respectively) [7]. Kurtosis for these activities in the general population occurred in the descending order of plasminogen, antithrombin, protein C, and protein S; thus, the distribution of protein S activity was the broadest. We also found age and gender differences for these activities. The activities of all proteins were significantly reduced in men older than 50 years. Decreases in protein C and protein S activity were particularly noticeable. Females in their 30's and 40's showed about 20% lower protein S activity than males. In particular, the plasma protein S activity can be influenced by many factors including age, gender, hormonal status, and disease [1,5,7,23]. Thus, the range of normal values for determination of risk can not be defined clearly, indicating the limitation of an activity assay for anticoagulant deficiency. DNA sequencing is an alternative approach to defining risk for DVT.

The sequencing of three genes revealed that the *PROS1* K196E mutation was the most prevalent (allele frequency of 0.049) in Japanese DVT patients. Mutations of *PROC* K193del and V339M were the second most prevalent in Japanese DVT patients, each found in four patients. The K193del and V339M mutations have been referred to as K150del or K151del and V297M, respectively, when the amino terminal Ala of the protein C light chain was designated as +1 [10,19,24]. These three deleterious mutations are concentrated in the Japanese population and contribute to the development of DVT. So far, the *PROS1* K196E and *PROC* K193del mutations have been found only in the Japanese population.

Table 7
Comparison of the first onset age of DVT between mutation carriers and non-carriers

	mutation carriers	non-carriers
number	55*	118
Onset age, year, mean±SD	44.7±16.5	52.6±16.1

*Five had mutations in *PROS1* and *PROC*. Two were homozygotes for *PROS1* Lys196Glu. One was a compound heterozygote for *PROS1* Lys196Glu and Arg101Cys.
p=0.0031.

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In the present study, we found that the protein C amidolytic activities in patients with the V339M mutation were generally lower than the normal level but those in patients with the K193del mutation were within the normal range (Table 4). It has been reported that the protein C anticoagulant activity in patients with the K193del mutation was actually low [10]. Therefore, we considered that the K193del mutation in the protein C molecule causes the loss of anticoagulant activity but not the amidolytic activity. We would suggest that the protein C amidolytic activity measurement might miss the identification of patients with K193del. The K193 residue is located at the sixth position from the C-terminus in the light chain of protein C and we cannot estimate how this residue functions in the protein C molecule.

We identified 38 nonsynonymous mutations in this study, 27 of which were missense mutations. In contrast to frameshift, splice-site, and nonsense mutations, the functional effects of missense mutations are not predictable [25]. Therefore, the effects of the genetic mutations identified in the present study might be overestimated. As shown in Table 4, some of the plasma samples of patients with the nonsynonymous mutations showed their activities within the normal range. A functional analysis of the mutants with missense mutations is needed for the definite elucidation of the deficiency. In particular, biochemical characterization of the protein C mutant with the second most prevalent mutation, K193del, is important.

Limitation of the study: There might have been a selection bias for recruitment of DVT patients. The patients in this study were not recruited consecutively. Patients with overt genetic deficiency of one of these three genes might be concentrated in our study population. Therefore, in a future study, we will need to recruit patients consecutively and classify DVT patients into unselected and selected groups to evaluate the contribution of genetic deficiency to DVT.

Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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