decreased by day 3, but remained more than 2-fold greater than normal for at least 7 days (Table 2), and this is evidence of persistent endothelial activation in sepsis. This also is consistent with previous studies that showed increased plasma VWF-pp level in sepsis and association with SOFA score and creatinine level but not with prognosis (24, 31).

The VWF-pp/ADAMTS13 ratio significantly correlated with disease severity including APACHE II score, SOFA score, the proinflammatory cytokine TNF-α, and creatinine during the period of observation (Table 3). Marked increase in the VWF-pp/ADAMTS13 ratio seemed to correlate with disease severity better than VWF-pp or ADAMTS13 level alone in patients with severe sepsis or septic shock. These results suggest that an imbalance between ULVWF secretion and ADAMTS13 level induced by endothelial activation or dysfunction may cause microthrombi and inflammation that lead to organ failure. In a porcine model of *Escherichia coli* sepsis, observations included decreased ADAMTS13 level, increased proportion of large-molecular-weight VWF multimers, glomerular microthrombi enriched with platelets and VWF, and acute renal failure (28). Therefore, the imbalance between VWF secretion and ADAMTS13 may induce platelet-VWF thrombosis in the kidney without appearance of ULVWF in plasma (29). Correcting this imbalance may help prevent or treat acute renal failure in sepsis.

We have recently found that ADAMTS13 may suppress intravascular growth of thrombus (32) and may control thrombosis and inflammation in the microcirculation in brain ischemia, brain reperfusion injury, and myocardial infarction (33–35). These suggested that administration of recombinant ADAMTS13 may correct the imbalance between ULVWF secretion and ADAMTS13 level and may help treat patients with severe imbalance who are at risk for multiple organ dysfunction. In children with thrombocytopenia, multiple organ dysfunction was resolved by restoring ADAMTS13 activity by plasma exchange (17). The VWF-pp/ADAMTS13 ratio may help identify patients with severe sepsis or septic shock at high risk for organ dysfunction because of imbalance between ULVWF secretion and ADAMTS13. Furthermore, this ratio may help identify patients susceptible for organ failure due to endothelial dysfunction in other diseases. Although the present prospective study was limited to few patients who had sepsis and thrombocytopenia, some trends were observed, and larger, controlled, prospective studies are necessary to evaluate and validate these findings.

CONCLUSION

The present study showed simultaneous changes in the levels of ADAMTS13, VWF-pp, VWF, and VWF-pp/ADAMTS13 ratio in patients during the first week of severe sepsis or septic shock. The ratio of VWF-pp/ADAMTS13 was associated with disease severity more than isolated VWF-pp or ADAMTS13 levels. Further studies may show whether organ failure may be prevented by identifying patients with abnormal VWF-pp/ADAMTS13 ratio and restoring the balance between VWF-pp and ADAMTS13 with plasma exchange or recombinant ADAMTS13.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Therapeutic Modality of 11 Patients with TTP in a Single Institution in Miyazaki from 2000 to 2011

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Abstract

Objective Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disease with pathological features that are termed thrombotic microangiopathies. Since the discovery of the von Willebrand factor-cleaving protease [a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13)], it is widely known that approximately two-thirds of TTP patients have a severe deficiency of ADAMTS13 activity due to gene mutations or acquired autoantibodies to this enzyme. However, the remaining one-third of TTP patients have only moderately reduced or almost normal ADAMTS13 activity. To elucidate the clinical characteristics and outcomes of these two types of TTP, we have retrospectively analyzed the cases of acquired TTP patients treated in a single institution from 2000 to 2011.

Methods Our case studies include 11 TTP patients, of which 5 were considered idiopathic and 6 had cases of TTP associated with underlying diseases such as non-Hodgkin lymphoma or connective tissue diseases.

Results These patients were treated with a combination therapy of plasma exchange and steroids and with several adjunctive therapeutic regimens including the on-label use of cyclophosphamide and cyclosporine and the off-label use of high-dose steroid or immunoglobulin with rituximab. Splenectomies were not performed. As a result of these treatments, 6 out of the 7 patients with ADAMTS13 activity deficient TTP achieved a complete remission without relapse, but the remaining 4 patients with non-ADAMTS13 activity deficient TTP all died without complete remission.

Conclusion We present herein the detailed clinical courses of 11 patients with TTP and address our experiences with the efficacy of various therapeutic regimens. This case-oriented study should be helpful to the physicians who directly care for TTP patients, and may provide a future direction for developing a more efficient treatment modality.

Key words: thrombotic thrombocytopenic purpura, ADAMTS13, plasma exchange, adjunctive therapeutic regimen, rituximab

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disease with pathological features that are termed thrombotic microangiopathies (TMAs). These features are characterized by the triad of microangiopathic hemolytic anemia, destructive thrombocytopenia, and organ (renal) failure due to platelet thrombi (1-5).

In 1996, Amorosi and Ultmann (2) defined the classic "pentad" of clinical features of TTP which included the aforementioned triad plus fluctuating neurological signs and

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fever. Regarding TTP treatment, Rock et al. (6) published a breakthrough report in 1991 showing that plasma exchange (PE) therapy saved the lives of 90% of TTP patients. However, the pathogenesis of TTP was not addressed until the discovery of the von Willebrand factor (VWF)-cleaving protease, now known as ADAMTS13-a disintegrin-like and metalloproteinase with a thrombospondin type I motifs 13 (7, 8). Subsequent studies have indicated that TTP is caused by severe deficiency of ADAMTS13 activity in approximately two-thirds of patients. Of these patients, a minor population (circa 5%) have the gene mutations which define Upshaw-Schulman syndrome (9, 10) and a major population (circa 95%) have acquired autoantibodies to this enzyme (11, 12). Therefore, the pathogenesis of TTP is not well defined and the efficacy of PE therapy has been controversial in the remaining one-third of acquired TTP patients who do not show severe deficiency of ADAMTS13 activity. Furthermore, recent studies indicate that some populations of acquired TTP patients with severe deficiency of ADAMTS13 activity do not respond well to PE therapy, and the reasons for this must be explored (13).

We treated 11 patients with acquired TTP from 2000 to 2011 in Miyazaki Prefectural Hospital, a regional referral hospital in Japan. In these patients, the basic PE and steroid therapy was supplemented with several adjunctive therapeutic regimens. These included the on-label use of cyclophosphamide and cyclosporine and the off-label use of high-dose steroid or immunoglobulin and rituximab, but did not include splenectomy. We analyzed the detailed clinical courses of our 11 TTP patients and evaluated the efficacy of the therapeutic regimens. This case-oriented study should be helpful to the physicians who directly care for TTP patients and may provide a future direction for the development of new treatment modalities.

Materials and Methods

Patients: From January 2000 to December 2011, 11 patients with acquired TTP were diagnosed at the Miyazaki Prefectural Hospital (Table 1). The diagnosis of TTP was made by the classic pentad with the following laboratory markers (14): (i) microangiopathic hemolytic anemia (hemoglobin [Hb] ≤12 g/dL), Coombs test negative, non-detectable serum haptoglobin (<10 mg/dL), more than 2 fragmented red cells (schistocytes) in a microscopic field with a magnification of 100, and the concurrent increase of serum lactate dehydrogenase (LDH) above the institutional baseline; (ii) thrombocytopenia (platelet count ≤100×109/L); (iii) fever ≥ 37℃; (iv) central nervous system (CNS) involvement ranging from headache to coma and including neurological dysfunction, convulsion, or clouding of consciousness; and (v) renal involvement including abnormal urinalysis in addition to the elevation of the serum creatinine level.

Among the 11 patients with TTP, 5 patients had no underlying disease and were classified as having acquired idiopathic TTP (ai-TTP) and 5 patients had TTP associated with connective tissue diseases. In this patient group, 2 had systemic lupus erythematosus (SLE), 2 had systemic sclerosis (SSc), and 1 had overlapping diseases (OS: SLE + SSc). The remaining patient had TTP associated with non-Hodgkin lymphoma (NHL) (Table 1). This retrospective study was conducted in compliance with good clinical practices and the ethical principles of the Declaration of Helsinki.

ADAMTS13 assays: Through March 2005, the ADAMTS 13 activity was measured at Nara Medical University by the classic von Willebrand factor multimer (VWFM) assay with a detection limit of 3% of the normal control (9). Thereafter, the ADAMTS13 activity was determined by a chromogenic ADAMTS13-act-ELISA (Chr-act-ELISA) with a detection limit of 0.5% of the normal (15). For consistency, all the samples tested prior to 2005 were re-evaluated by Chr-act-ELISA using the plasmas that had been stored at -80℃.

The plasma ADAMTS13 inhibitor titers were analyzed using patient plasmas that had been heat-inactivated at 56℃ for 30 minutes (15) according to the original method established for the measurement of factor VIII inhibitor (16). The results were expressed in Bethesda units (BU) where one unit was defined as the amount necessary to reduce the ADAMTS13 activity to 50% of the control levels. The ADAMTS13 inhibitor titers were considered negative for values of less than 0.5 BU/mL, marginal for values between 0.5 and 1 BU/mL, and positive for values greater than 1 BU/mL (14).

Plasma exchange therapy: We performed plasma exchange (PE) therapy with fresh frozen plasma (FFP) at 60 mL/kg body weight until we observed the recovery of the following variables: increased platelet count (>150×10^9/L), decreased lactate dehydrogenase (LDH) levels, and decreased neurological abnormalities (4, 8). The PE therapy was performed for 3 consecutive days from the diagnosis of TTP and then the frequency was gradually tapered off. A diagnosis of complete remission (CR) was considered when a normalization of both the physical and the laboratory findings were achieved as previously described (4). The PE therapy was usually accompanied by high-dose methylprednisolone (mPSL) pulse therapy with an intravenous drip infusion rate of 1 g mPSL/day for 3 consecutive days.

Case series: The clinical and laboratory findings at admission for 11 patients with acquired TTP are shown in Table 1 and their treatment regimens and therapeutic outcomes are summarized in Table 2.

Case 1: In 2003, a 56-year-old man developed petechiae and a fever, then fell into a coma prior to his admission to our hospital. Based on the clinical diagnosis of TTP, PE therapy was immediately initiated with high-dose mPSL pulse therapy under sedation and with intubation. The patient was extubated on hospital day 13 but the thrombocytopenia remained, and, thus, a second course of mPSL pulse therapy was initiated on hospital day 14. On hospital day 16, the laboratory results showed that a low plasma level of ADAMTS13 activity (<3%) and positivity for an ADAMTS

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Table 1. Clinical and Laboratory Findings on Admission of Eleven Patients with Acquired TTP

Case			1	2	3	4	5	6	7	8	9	10	11
Gender			M	F	M	M	F	F	F	F	F	M	F
Age			56	50	48	56	50	17	71	68	56	59	51
Body Weight (kg)			65	45	70	70	55	50	40	48	55	50	71
Etiology			Idiopathic	Idiopathic	Idiopathic	NHL	OS (SLE+SSc)	SLE	Idiopathic	SSc	SSc	Idiopathic	SLE
Year			2003	2005	2007	2007	2007	2008	2008	2008	2008	2009	2009
Initial clinical signs													
Fever (°C)			39.0	38.6	37.2	36.0	38.1	37.8	38.6	38.8	38.0	38.8	38.1
Anemia			+	+	+	+	+	+	+	+	+	+	+
Purpura			+	+	+	+	+	+	+	+	+	+	+
Renal disfunction			-	-	-	+	+	-	+	+	+	+	-
Neurologic signs			Coma (JCS 200)	Coma (JCS 200)	Right Hemiplegia, eye deviation to the right	(JCS 30)	Tonic-clonic convulsion	Tonic-clonic convulsion	(JCS 200)	(JCS 30)	Right hemiplegia	Coma (JCS 200)	Coma (JCS 200)
Peripheral blood		Normal ranges											
Platelets	($\times10^9$/L)	137-378	11.0	11.0	7.0	7.0	32.0	6.0	8.0	86.0	29.0	14.0	15.0
WBC	($\times10^9$/L)	3.0-8.7	6.3	10.4	10.2	5.2	7.6	3.8	7.4	8.1	12.4	12.9	5.9
RBC	($\times10^{12}$/L)	3.7-4.9	2.5	2.0	2.5	2.0	3.4	2.2	2.3	3.6	2.6	2.7	3.4
Hb	(g/dL)	10.7-15.3	8.6	6.6	7.9	6.4	9.1	6.7	7.6	9.1	7.6	6.2	9.1
Reticulocyte	(‰)	0.4-1.6	6.1	12.6	9.4	3.4	8.2	7.5	4.1	1.1	3.2	6.2	4.7
Schistocytes on blood film		-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Blood chemistry													
Total protein	(g/dL)	6.7-8.3	6.6	6.2	7.6	5.5	6.9	8.3	5.8	6.1	4.2	7.2	7.6
Total bilirubin	(mg /dL)	0.2-1.2	3.5	2.8	4.3	0.6	0.7	2.4	6.5	0.7	1.7	2.1	4.2
Direct bilirubin	(mg /dL)	0-0.2	0.5	0.2	0.4	0.2	0.2	0.4	1.1	0.3	0.4	0.4	0.4
AST	(IU/L)	7-38	47	36	43	17	217	33	364	42	80	37	41
ALT	(IU/L)	4-43	30	15	29	8	63	15	220	20	20	21	20
LDH	(IU/L)	119-229	910	753	1,295	415	972	756	1,730	1,035	3,750	1,138	947
BUN	(mg /dL)	8.0-20.0	16	14	24	18	69	9	36	33	60	36	29
Creatinine	(mg /dL)	0.4-0.8	1.1	1.2	1.3	1.6	2.6	0.6	2.0	2.1	1.6	1.4	1.0
CRP	(mg /dL)	0-0.3	1.5	0.1	0.8	0.1	2.5	0.2	4.2	2.9	0.6	3.0	0.3
Haptoglobin	(mg /dL)	19-170	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Hemostatic test													
PT	(sec)	9.3-12.0	12.0	12.0	14.0	10.8	9.7	13.0	13.6	12.7	13.7	12.8	12.3
A-PTT	(sec)	24.0-40.0	34.3	38.3	35.1	30.3	36.3	32.3	33.0	35.3	35.3	34.6	25.4
Fibrinogen	(mg /dL)	150-360	168	174	327	361	300	194	167	230	210	154	247
Antithrombin III	(%)	70.0-130.0	81	105	96	87	88	92	78	82	92	102	94
FDP-P	(μg/mL)	0-5.0	4.0	6.0	3.2	1.3	7.0	2.0	4.8	6.0	7.0	4.9	7.7
D-dimer	(μg/mL)	0-1.0	2.0	3.1	2.0	0.3	4.1	1.0	2.1	3.2	3.6	2.2	4.0
PA IgG	(ng/10^7cells)	<46	96.3	220.8	53.7	72.0	129.0	52.0	65.8	75.8	89.0	196.0	86.0
ADAMTS 13													
Activity													
VWFM	(%)		<3.0	<3.0	<3.0								
Chr-ELISA	(%)	70-120	<0.5	<0.5	<0.5	39	49	<0.5	<0.5	47	69	<0.5	<0.5
Inhibitor													
VWFM	(BU/mL)		1.8	1.6	1.3								
Chr-ELISA	(BU/mL)	<0.5	1.6	2.1	2.2	ND	ND	1.4	1.1	ND	ND	2.0	2.2

Among the 11 patients with TTP, 5 had no underlying diseases and were termed acquired idiopathic TTP (ai-TTP) and 5 had TTP that could be associated with connective tissue diseases. Of this second group, 2 patients had systemic lupus erythematosus (SLE), 2 patients had systemic sclerosis (SSc), and 1 patient had overlapping disease (OS: SLE + SSc). The remaining non-idiopathic patient had non-Hodgkin lymphoma (NHL).

Table 2. Treatment and Therapeutic Outcomes on Eleven Patients with Acquired TTP

Case	1	2	3	4	5	6	7	8	9	10	11
Initial Therapy											
Intubation Extubation	HD 1 HD 13	HD 1 HD 10	HD 1 HD 8	- -	HD 3 HD 10	HD 3 HD 5	HD 1 HD 16	HD 7 HD 13	HD 1 HD 3	HD 1 HD 10	HD 1 HD 7
Plasma Exchange (PE)	30 times	19 times	12 times	9 times	14 times	6 times	10 times	8 times	2 times	13 times	14 times
Methyl prednisolone pulse	HD 1-3 and HD 14-16	HD 1-3	HD 1-3 and HD 11-13	HD 7-9	HD 3-5	HD 3-5	HD 1-3	HD 7-9	HD 1- 2	HD 1-3	HD 1-3
Prednisolone (1mg/kg)				HD 1-6	HD 1-3			HD 1-6			
Additional Treatment											
Intravenous γ-globulin (a dose of 400 mg/kg)	HD 49-53	HD 21-25	HD 15-19								
Cyclosporine (4-5 kg/m^2)							HD 15 - 28			HD 20 - the present time	
Vincristine (a dose of 1mg/m^2)	HD 21,28, 35 and 42			HD 15, 22, 29 and 36							
Cyclophosphamide pulse (500 mg/m^2)					HD 15			HD 14			HD 20
Rituximab (a dose of 375 mg/m^2)				HD 29, 36, 42 and 49		HD 13, 20, 27 and 34		HD 21,28,35 and 42			
Maintenance Treatment											
Prednisolone Cyclosporine	5mg	5mg	5mg	-	-	5mg	-	-	-	5mg 50mg	5mg
Response	CR	CR	CR	no response	PR	CR	PR	PR	no response	CR	CR
Outcome	DFS	DFS	DFS	dead (HD 76)	dead (HD102)	DFS	dead (HD 28)	dead (HD 56)	dead (HD 3)	DFS	DFS

(HD : hospital days, CR : complete remission, PR : partial remission, DFS : disease-free survival)

All 11 patients were treated with this combination regimen of PE and high-dose PSL therapy. Some of the patients were also treated with the following adjunctive therapies: high-dose IVIG, cyclosporine, vincristine, cyclophosphamide, and rituximab.

13 inhibitor (1.8 BU/mL) were present on admission, confirming the diagnosis of ai-TTP with a severe deficiency of ADAMTS13 activity. Because thrombocytopenia persisted (10×10^9/L), 4 cycles of vincristine at a dose of 1.0 mg/m^2 per week were initiated starting on hospital day 21. Moreover, an intravenous infusion of gamma globulin (IVIG) was administered at a dose of 400 mg/kg for 5 consecutive days starting on hospital day 49 because of persistent thrombocytopenia (25×10^9/L). After these treatments, combined with 30 rounds of PE therapy, a CR was achieved on hospital day 63. To date, the patient maintains disease-free survival (DFS) with an oral intake of 5 mg PSL/day.

Case 2: In 2005, a 50-year-old woman developed petechiae and a fever, and thereafter fell into a coma prior to her admission to our hospital. Based on the clinical diagnosis of TTP, PE therapy was immediately initiated with high-dose mPSL pulse therapy under sedation and with intubation. The patient was extubated on hospital day 10 because her platelet count increased to 100×10^9/L. On hospital day 12, the laboratory findings revealed that a low plasma level of ADAMTS13 activity (<3%) and positivity for an ADAMTS 13 inhibitor (1.6 BU/mL) were present at admission, confirming the diagnosis of ai-TTP with a severe deficiency of ADAMTS13 activity. Because clinical aggravations were observed while tapering off the administration of PE and PSL, a high-dose IVIG treatment was administered for 5 consecutive days starting on hospital day 21. A CR was achieved on hospital day 40 after 19 rounds of PE therapy. To date, the patient maintains DFS with an oral intake of 5 mg PSL/day.

Case 3: In 2007, a 48-year-old man developed a fever with subsequent eye deviation to the right and right hemiplegia prior to his admission to our hospital. Based on the clinical diagnosis of TTP, PE therapy was immediately initiated with high-dose mPSL pulse therapy under sedation and with intubation. The patient was extubated on hospital day 8. On hospital day 11, laboratory findings revealed that a low plasma level of ADAMTS13 activity (<3%) and positivity for an ADAMTS13 inhibitor (1.3 BU/mL) were present on admission, confirming the diagnosis of ai-TTP with a severe deficiency of ADAMTS13 activity. Because the clinical and laboratory findings were exacerbated when the PE therapy was tapered off, a second course of high-dose mPSL pulse therapy was initiated starting on hospital day 11. In addition, a high-dose IVIG treatment was administered for 5 consecutive days starting on hospital day 15, which remarkably improved the neurological and hematological findings. A CR was achieved on hospital day 42 after 12 rounds of PE therapy. Relapse has not been observed to date with an oral intake of 5 mg PSL/day. However, the patient did develop osteonecrosis of the right femur requiring an anterior rotation osteotomy in April 2011 which may have been associated with an adverse reaction to the two courses of high-dose mPSL pulse therapy.

Case 4: In 2007, a 56-year-old man developed petechiae without a fever and subsequently developed anemia and thrombocytopenia prior to his admission to our hospital. The clinical and laboratory findings upon admission suggested a diagnosis of TTP, but this patient also had a history of non-Hodgkin lymphoma (NHL) with CS IIIA at the age of 54. He achieved a CR from the NHL after 8 courses of treatment with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) therapy, but relapsed at the age of 55. However, he achieved a second CR after being treated in December 2006 with salvage therapy and

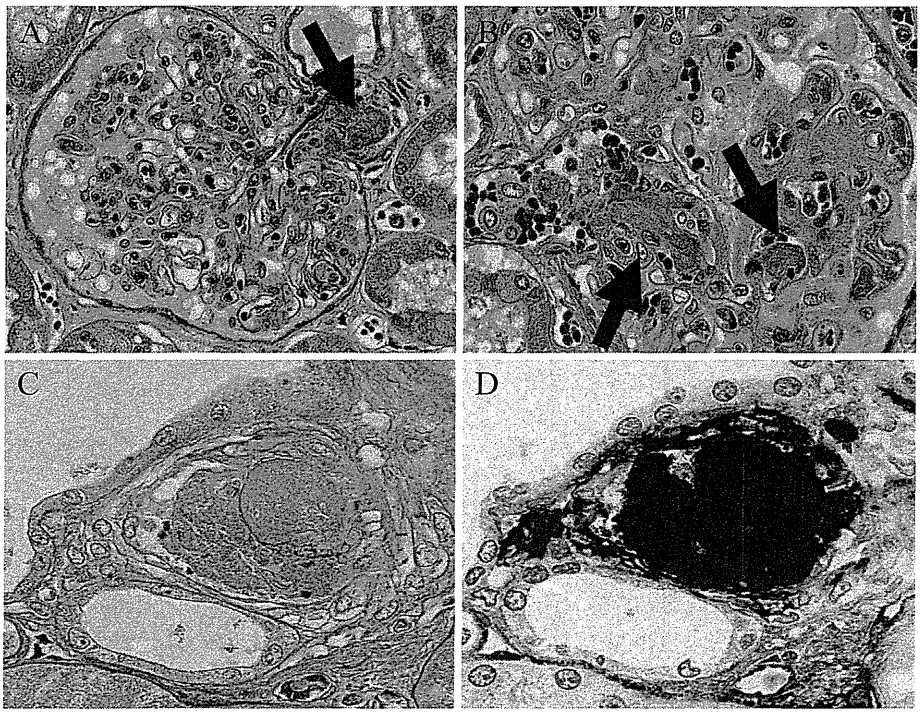

Figure. A post-mortem histological examination of a renal specimen from a patient with non-ADAMTS13 activity deficient TTP (See Case 5). Both Figures A and B show Hematoxylin and Eosin staining, and the arrows indicate platelet thrombi. Figure C is the immuno-staining with the anti-fibrinogen polyclonal antibody (DAKO, Glostrup, Denmark), and Figure D is the immune-staining with the anti-von Willebrand factor (VWF) polyclonal antibody (DAKO, Glostrup, Denmark). Note that the VWF-rich platelet thrombi in this patient show a sharp contrast to those in patients with disseminated intravascular coagulation (DIC) (17).

high-dose chemotherapy followed by an autologous peripheral blood stem cell transplantation (PBSCT). Because of his clinical background, he was initially treated with oral PSL (1 mg/kg) in 2007, but his condition soon worsened and he fell into a coma on hospital day 7. Under a clinical diagnosis of PBSCT-associated TTP, he received PE therapy with high-dose mPSL therapy starting on hospital day 7. On that day, the laboratory findings revealed that a slightly decreased plasma level of ADAMTS13 activity (39%) and negativity for an ADAMTS13 inhibitor were present on admission. Despite treatments with PE and mPSL therapy, his platelet count did not increase. For this reason, 4 cycles of vincristine administered at a dose of 1.0 mg/m^2 per week were initiated starting on hospital day 15, but the patient failed to improve. Four additional cycles of rituximab administered at a dose of 375 mg/m^2 per week were initiated starting on hospital day 29, but did not improve his clinical condition. He died of sepsis due to methicillin-resistant Staphylococcus aureus (MRSA) on hospital day 76 after receiving PE therapy 6 times.

Case 5: In 2007, a 50-year-old woman developed a fever, general fatigue, and dermatitis. She was admitted to our hospital after her family doctor found evidence of anemia and thrombocytopenia. Based on her low serum levels of complement, the presence of a skin rash, and the presence of anti-nuclear antibodies (ANA), the anti-ds DNA antibody, the anti-Sm antibody, and the anti-Scl 70 antibody, she was diagnosed with OS (SLE and SSc) and was treated with oral PSL (1 mg/kg). However, she developed the neurological symptoms of coma and tonic-clonic convulsions on hospital day 3. Taken together, these findings indicated a diagnosis of OS-associated TTP. PE therapy was initiated with high-dose mPSL therapy in parallel with continuous hemodialysis under sedation and with intubation from hospital day 3. The patient was extubated on hospital day 10. On that same day, laboratory findings revealed that a slightly decreased plasma level of ADAMTS13 activity (49%) and negativity for an ADAMTS13 inhibitor were present on admission. The exacerbation of the neurological symptoms and thrombocytopenia were observed while tapering off the administration of PE and PSL. Thus, cyclophosphamide (500 mg/m^2) was administered starting on hospital day 15 which resulted in a transient rise in her platelet count. A partial remission (PR) was achieved on hospital day 44 after 14 rounds of PE therapy. However, the patient died of an uncontrolled progression of aspergillus pneumonia on hospital day 102 despite the administration of liposomal amphotericin B. During the autopsy, platelet thrombi stained with the anti-VWF polyclonal antibody were detected in her kidney, heart, brain, and intestine (Figure). Since platelet thrombi in the microvasculatures that are rich in VWF have been hallmarks of ADAMTS13 activity deficient TTP (17), it was interesting

to note that the same pathological change was observed in this instance of non-ADAMTS13 activity deficient TTP. This finding indicates that both the severe deficiency of ADAMTS13 activity and a moderate deficiency with an extremely low ratio of ADAMTS13:Unusually Large VWF Multimers (UL-VWFM) lead to the same pathological results.

Case 6: In 2008, a 17-year-old female patient developed a fever, general fatigue, headache, vomiting, and arthralgia. She was admitted to our hospital after her family doctor found evidence of anemia and thrombocytopenia. She also had Raynaud's phenomenon, low serum levels of complement, and was positive for ANA, the anti-ds DNA antibody, and the anti-Sm antibody. These findings indicated a diagnosis of SLE and she was treated with PSL (1 mg/kg). She developed the neurological symptom of tonic-clonic convulsions on hospital day 3. By this point, she had been diagnosed with SLE-associated TTP, and PE therapy with high-dose mPSL pulse therapy under sedation and with intubation was instituted starting on hospital day 3. The patient was extubated on hospital day 5 after her platelet count increased (70×10^9/L). On hospital day 7, the laboratory findings revealed that a low plasma level of ADAMTS13 activity (<0.5%) and positivity for an ADAMTS13 inhibitor (1.4 BU/mL) were present on admission, confirming a diagnosis of SLE-associated acquired TTP with a severe deficiency of ADAMTS13 activity. Her neurological symptoms worsened on hospital day 12 despite intensive PE and mPSL therapy. As a result, an off-label treatment with rituximab was initiated starting on hospital day 13 which remarkably improved the abnormal clinical and laboratory findings. A CR was achieved on hospital day 45 after 6 rounds of PE therapy. To date, the patient maintains DFS with an oral intake of 5 mg PSL/day.

Case 7: In 2008, a 71-year-old woman developed a fever and chest pain and soon fell into a coma prior to being transferred to our hospital. The findings of abnormal electrocardiogram, elevated cardiac enzymes, and the asynergy of the antero-septal wall by ultrasound cardiogram confirmed a cardiac failure due to acute myocardial infarction (AMI). Due to her cardiac condition, PE therapy with a reduced amount of FFP (30 mL/kg) was initiated with high-dose mPSL therapy under sedation and with intubation starting on hospital day 1 in parallel with continuous hemodialysis. The laboratory findings on hospital day 9 revealed that a low plasma level of ADAMTS13 activity (<0.5%) and positivity for an ADAMTS13 inhibitor (1.1 BU/mL) were present on admission, thus confirming a diagnosis of ai-TTP with a severe deficiency of ADAMTS13 activity. Worsening clinical signs of acute cardiac failure and pulmonary edema necessitated the cessation of PE therapy after 10 rounds. Instead, cyclosporine therapy at a dose of 4 mg/day was started on hospital day 15. Although these treatments resulted in a partial remission (PR) for TTP, the patient died of cardiac shock associated with her AMI on hospital day 28.

Case 8: In 2008, a 68-year-old woman with dermatitis and chronic heart failure was diagnosed with anemia and thrombocytopenia by her family doctor and admitted to our hospital. Due to the presence of a skin rash, a positive ANA result, and the presence of the anti-Scl 70 antibody, she was diagnosed with SSc and was treated with PSL (1 mg/kg). During the treatment period, she went into a coma and experienced a pulmonary hemorrhage on hospital day 7. She was subsequently diagnosed with SSc-associated TTP. PE therapy at a reduced volume of FFP (30 mL/kg) with high-dose mPSL therapy was initiated in parallel with continuous hemodialysis with intubation starting on hospital day 7. The patient was extubated on hospital day 13. On that same day, the laboratory findings revealed that a slightly decreased plasma level of ADAMTS13 activity (47%) and negativity for an ADAMTS13 inhibitor were present on admission. The neurological symptoms and pulmonary hemorrhage were exacerbated when the PE and PSL administration were tapered off. As a result, we added cyclophosphamide (500 mg/m^2) pulse therapy on hospital day 14, but the patient failed to improve. We subsequently initiated off-label therapy with rituximab starting on hospital day 21. This treatment transiently improved the clinical signs and a PR was achieved on hospital day 42 after 8 rounds of PE therapy. However, the progression of a nosocomial aspergillus pneumonia could not be controlled despite the use of amphotericin B, and the patient died on hospital day 56.

Case 9: In 2008, a 56-year-old woman visited a local physician who identified dermatitis of Gottron's sign, arthritis, and the presence of ANA and the anti-SCL70 antibody. She was diagnosed with SSc and treated with PSL (1 mg/kg). Soon after, she developed anemia and thrombocytopenia and fell into a coma with cerebral hemorrhage. Simultaneously, she had lung lesions indicative of interstitial pneumonia with pulmonary hemorrhage. Due to these serious clinical conditions, she received a platelet transfusion with a dose of 10 units (2×10^{11} platelets) after which her clinical signs worsened and necessitated her transfer to our hospital. Clinical and laboratory findings upon admission indicated a clinical diagnosis of SSc-related TTP. The patient immediately received PE therapy for 2 consecutive days concurrently with high-dose mPSL pulse therapy. However, she died from the progression of a cerebral hemorrhage on hospital day 3 without appreciable clinical improvements. After her death, laboratory findings revealed that a normal plasma level of ADAMTS13 activity (69%) and negativity for an ADAMTS13 inhibitor were present on admission.

Case 10: In 2009, a 59-year-old man developed a fever, diarrhea, and gastrointestinal hemorrhage. The patient subsequently fell into a coma with left hemiplegia and was admitted to our hospital. PE therapy was initiated with high-dose mPSL pulse therapy after sedation and with intubation on hospital day 1. The patient was extubated on hospital day 10 because his clinical signs had improved. On hospital day 10, laboratory findings revealed that a deficient plasma ADAMTS13 activity (<0.5%) with positivity for an

ADAMTS13 inhibitor (2.0 BU/mL) were present upon admission, thus confirming a diagnosis of ai-TTP with severe deficiency of ADAMTS13 activity. However, exacerbation of the thrombocytopenia was observed when PE and PSL administrations were tapered off. Thus, cyclosporine (5 mg/kg) was administered starting on hospital day 20 which resulted in an increased platelet count. A CR was achieved on hospital day 35 after 13 rounds of PE therapy. To date, the patient maintains DFS with an oral intake of PSL 5 mg/day.

Case 11: In 2009, a 51-year-old woman developed a fever and sore throat and subsequently fell into a coma prior to her transfer to our hospital. In addition, she had a skin rash, low serum levels of complement, and was positive for ANA, the anti-ds DNA antibody, and the anti-Sm antibody. On the basis of these additional findings, she was diagnosed with SLE-associated TTP. PE therapy was initiated immediately with high-dose mPSL therapy under sedation and with intubation starting on hospital day 1. These treatments rapidly improved her laboratory and clinical findings, and she was extubated on hospital day 7. On hospital day 9, the laboratory findings revealed a low plasma level of ADAMTS13 activity (<0.5%) and positivity for an ADAMTS13 inhibitor (2.2 BU/mL), confirming the diagnosis of SLE-associated TTP with a severe ADAMTS13 deficiency. The neurological symptoms and thrombocytopenia were exacerbated when the PE and PSL administrations were tapered off. Thus, cyclophosphamide (500 mg/m^2) pulse therapy was added starting on hospital day 20, which gradually improved thrombocytopenia. A CR was achieved on hospital day 32 after 14 rounds of PE therapy. The patient maintains DFS with an oral intake of 5 mg PSL/day.

Case series summary: Of our 11 patients, 7 had ADAMTS13 activity-deficient TTP and 4 had non-ADAMTS13 activity-deficient TTP. Although idiopathic TTP develops with a fever in the absence of any known etiology, TTP can also be associated with various underlying diseases. Of the 11 patients included in our study, 5 were idiopathic and 6 had cases that were associated with underlying causes such as non-Hodgkin lymphoma (n=1) and connective tissue diseases (n=5) including SLE, SSc, and OS.

Regarding the clinical pentad in TTP, all of our 11 patients had a fever, hemolytic anemia, thrombocytopenia, and neurological signs upon admission, but 5 lacked renal dysfunction (Table 1). The laboratory findings indicated that the platelet counts and serum LDH levels were highly variable, but the serum haptoglobin levels were uniformly very low (<10 mg/dL). Moreover, non-ADAMTS13 activity-deficient TTP patients had tendency to present with high values of LDH and low values of bilirubin. With regard to ADAMTS 13, 7 of our patients had ADAMTS13 activity-deficient TTP with a low titer of ADAMTS13 inhibitors (1.1-2.2 BU/mL), whereas the remaining 4 had a slightly reduced or almost normal ADAMTS13 activity levels without detectable inhibitors.

Of the 11 patients treated for TTP, 6 are alive with a mean survival time of 1,014.6±1,101.0 days (mean ± SD). The survival rates of ADAMTS13 activity-deficient TTP patients and non-ADAMTS13 activity-deficient TTP patients were 85.7% and 0%, respectively, and the respective mean survival times were 1,816.0±853.3 days and 53.0±38.9 days.

The causes of death identified during this study included cardiogenic shock due to heart failure (patient 5), aspellugillus pneumonia (patients 8 and 10), MRSA sepsis (patient 9) and cerebral hemorrahage (patient 11). Patients with idiopathic, ADAMTS13 activity-deficient TTP did not develop any other diseases such as collagen diseases during the follow-up periods.

Discussion

The detailed clinical and laboratory findings were well preserved in this study because all 11 patients with TTP were treated in a single institution at Miyazaki during the past 12 years. Of these 11 patients, 5 had idiopathic TTP and 6 had cases of TTP that were associated with underlying issues such as non-Hodgkin lymphoma or connective tissue diseases. Regarding the clinical pentad of TTP, all 11 of the patients had a fever, hemolytic anemia, thrombocytopenia, and neurological signs upon admission, but 5 lacked renal dysfunction (Table 1). Although the platelet counts and serum LDH levels were highly variable between patients, their serum haptoglobin levels were uniformly very low (<10 mg/dL). Of these 11 patients, 7 had ADAMTS13 activity deficient TTP with low-titer ADAMTS13 inhibitors (1.1-2.2 BU/mL), whereas the remaining 4 had a slightly reduced or almost normal ADAMTS13 activity without any detectable inhibitors. The non-ADAMTS13 activity deficient TTP patients tended to display high LDH values and low bilirubin values. Notably, the plasma levels of fibrin degradation products (FDP)-P and D-dimer were slightly elevated in 3 out of the 4 non-ADAMTS13 activity-deficient TTP patients. These findings suggested that non-ADAMTS13 activity deficient TTP patients who suffer from an underlying disease are prone to suffer from a fibrinogen consumption commonly known as disseminated intravascular coagulation (DIC). Habe et al. (18) recently reported that ADAMTS13/VWF profiles may have important roles in the pathogenesis of DIC, and that the plasma levels of both ADAMTS13 and VWF propeptide are useful indicators for the diagnosis and prognosis of DIC. In contrast, the marker for immune thrombocytopenia (ITP), platelet-associated (PA) IgG, was present in significantly increased levels in all of our TTP patients, thereby indicating that PAIgG is not useful for differentiating between ITP and TTP.

Of our 11 patients with TTP, 6 are still alive. The survival rates of ADAMTS13 activity-deficient TTP patients and non-ADAMTS13 activity-deficient TTP patients were 85.7% and 0%, respectively, and the respective mean survival times were 1816.0±853.3 days and 53.0±38.9 days. Statistically significant differences between these two groups were observed in the incidence of renal dysfunction (p=0.02) and in

the total bilirubin counts (p=0.002).

Since 1991, PE therapy with or without an adjunctive regular or high-dose (pulse) steroid therapy has been used as the first-line treatment of TTP (6, 19-25). The discovery of ADAMTS13 allowed the efficacy of PE therapy in TTP patients to be quantifiably analyzed. Normal hemostasis can now be achieved by the replenishment of ADAMTS13 and regular-sized VWFM in addition to the removal of hazardous materials such as UL-VWFM, anti-ADAMTS13 autoantibodies, and the elevated inflammatory cytokines that upregulate UL-VWFM release from vascular endothelia cells. All 11 of our patients were treated with the combination regimen of PE therapy with high-dose mPSL pulse therapy because ADAMTS13 data were not readily available upon admission. The patient group received PE therapy between 6 and 30 times, and none of the patients showed a rebound or increase in their inhibitor titers during their PE therapy and subsequent clinical courses. The high-dose mPSL pulse therapy administered in tandem aimed to sedate the severe, fluctuating neurological signs associated with TTP as well as to suppress the anti-ADAMTS13 antibody production in patients with ADAMTS13 activity-deficient TTP. Ito-Habe et al. (26) report that a high-dose mPSL pulse therapy (1 g/day) is now administered in the majority of Japanese institutions rather than standard PSL therapy (1 mg/kg). According to their response to the combination regimen of PE therapy and mPSL pulse therapy, some of our patients were treated with the following adjunctive therapies: high-dose IVIG, cyclosporine, vincristine, cyclophosphamide, or rituximab. A few of these adjunctive drugs now have well-documented efficacies based on pharmacokinetics. The adjunctive therapies were administered based on the underlying disease, the unrecovered clinical symptons, the unresolved laboratory data, and the degree of organ dysfunction. We tended to administer rituximab for the refractory setting of TTP after the drug became available for off-label use in 2007. Due to our therapeutic regime, 6 out of the 7 patients with ADAMTS13 activity-deficient TTP accomplished CR with the exception from this group being the case that died from a complication of her AMI. In contrast, all 4 of the non-ADAMTS13 activity deficient TTP patients died.

While a few studies documented the efficacy of high-dose IVIG therapy in TTP patients prior to the discovery of ADAMTS13, subsequent studies could not confirm this finding (27, 28). Between 2003 and 2007, we used high-dose IVIG therapy at a dosage of 400 mg/kg IVIG for 5 consecutive days in 3 patients with ADAMTS13 activity-deficient TTP (cases 1-3) as an adjunctive therapy when the clinical aggravation of thrombocytopenia and persistent thrombocytopenia were observed during the tapering off period of the PE and PSL therapies. Interestingly, all the patients showed a good response to high-dose IVIG therapy. However, as described below, rituximab is now preferred over high-dose IVIG therapy for the treatment of TTP patients with relapsing and intractable clinical courses. If the efficacy of high-dose IVIG is proven on a scientific basis in the future, this therapy might be revived.

Among the adjunctive therapies, rituximab treatment may be most reasonable and powerful therapeutic modality for depleting the B cells that produce the autoantibodies for ADAMTS 13. Several reports in the literature have clearly shown that rituximab, an anti-CD20 chimeric monoclonal antibody, is highly efficient as an adjunctive therapy for TTP patients who do not respond adequately to a standard combination therapy of PE and steroids (29-31). Globally, the on-label therapeutic use of rituximab is for CD20-positive malignant B-cell lymphoma. Because the B-lymphocyte is an IgG antibody-producing cell, a broad spectrum of autoimmune diseases and associated symptoms can be targeted by rituximab, including the ADAMTS13 deficiency in TTP patients due to the presence of autoantibodies. The most recent study indicates that rituximab administration at a dosage of 375 mg/m^2 weekly for 4 cycles almost completely depletes the circulating B-lymphocytes from 16 hospital days to 3 months (32). Three of our TTP patients (cases 4, 6, and 8) were treated with rituximab between 2007 and 2008. The patient with ADAMTS13 activity deficient TTP (Case 6) due to a low titer of ADAMTS13 inhibitor (1.4 BU/mL), responded well to this treatment and has maintained DFS to date. However, the two patients with non-ADAMTS13 activity deficient TTP (Cases 4 and 8) died on hospital days 76 and 56, respectively. We therefore did not find any benefit in rituximab therapy for patients with non-ADAMTS13 activity-deficient TTP. However, of our 7 patients with ADAMTS13 activity-deficient TTP due to its inhibitors (Cases 1, 2, 3, 6, 7, 10, and 11), Case 6 alone was treated with rituximab and had a favorable outcome. During her course of treatment, Case 6 received less PE therapy than the other cases (6 rounds in Case 6 versus 9 to 30 times in our other cases), suggesting that the adjunctive use of rituximab has a plasma-sparing effect that must be confirmed in future studies. Scully et al. (32) reported in a Phase 2 study that rituximab treatment with PE was a safe and effective treatment for ai-TTP patients, thus validating our observations. Kameda et al. (33) have recently reported that rituximab treatment may be effective for non-ADAMTS 13 activity deficient TTP patients, but we did not confirm this to be true for our study. Their results may be explained by the reduction of excessive cytokine production through the rituximab-driven B cell depletion in non-ADAMTS13 activity deficient TTP patients, but that hypothesis needs to be carefully evaluated in future studies. We are presently unable to discuss the efficacies of the additional adjunct therapies, including including vincristine, high-dose cyclophosphamide, and cyclosporine, due to their low administration frequency to patients in this study.

In conclusion, we believe that this case-oriented study will be highly useful to the physicians who directly care for TTP patients.

The authors state that they have no Conflict of Interest (COI).

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Naoko Yokota-Ikeda equally contributed to this work.

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LETTER TO THE EDITOR
Limited Renal Prophylaxis in Regular Plasmatherapy for Heritable ADAMTS13 Deficiency

To the Editor: Germline mutations of *ADAMTS13* cause chronic relapsing-thrombotic thrombocytopenic purpura (TTP), called Upshaw–Schulman syndrome. Affected patients with the rare disease may develop renal impairment with a need for dialysis unless thrombotic microangiopathy is controlled [1]. Plasma exchange is the standard therapy for disease control. There is little information about the long-term effects of plasmatherapy on renal disease. We report the outcomes of patients who underwent >15-years' regular plasma infusion.

Three unrelated patients were treated in our hospitals (Table I) [2]. All presented the classical triad of neonatal jaundice, hemolytic anemia, and thrombocytopenia requiring whole-blood exchange transfusion. Chronic relapsing-TTP was diagnosed in Pt-1, Pt-2, and Pt-3 at age 16 months, 7 years, and 1 month, respectively. Pt-1 suffered a varicella-induced TTP at age 7 months. On-demand infusion of plasma-derived intermediate purity factor VIII concentrates, Confact F (Kaketsuken, Kumamoto, Japan) or Conco-eight (Green Cross Corp., Osaka, Japan) was the first line treatment [3,4]. In Japan, they were replaced by monoclonal antibody-purified/recombinant FVIII products, therefore, not appropriate replacement. Pathogen-inactivated plasma was unavailable. Regular infusion of fresh-frozen plasma (FFP) was then started to control repetitive bouts. No IgG-antibody against ADAMTS13 was detected in any patient.

The FFP prophylaxis and current renal functions are shown in Table I. At the time of infusion, all patients showed petechiae, thrombocytopenia at around 10×10^9/L, and non-severe hemolysis represented by changing hemoglobin, total bilirubin, and lactate dehydrogenase levels, along with proteinuria and hematuria/hemoglobinuria. However, stable hematological responses led to no hospitalization in three patients. Two patients had anti-hepatitis C virus (HCV) antibody, but not HCV RNA or abnormal transaminase levels. Pt-1 had occasional infusion reactions including anaphylaxis. No immunologic disorders developed. Serum concentrations of creatinine ranged normally in Pt-1 and Pt-2, but increased in Pt-3 beyond 20 years of prophylaxis. All had <90 ml/min/1.73 m^2 of the estimated glomerular filtration rate (eGFR), that was calculated by the formula [5]. Pt-3 had high urine protein to creatinine ratio. At the time of FFP infusion, Pt-3 always showed hemoglobinuria, hematuria, proteinuria, and granular casts, Pt-1 had proteinuria only, and Pt-2 showed neither. Pt-1 accepted to undergo the renal biopsy at age 9 years, histopathology of which demonstrated a few microthrombotic glomeruli but no significant inflammation (Supplemental Fig. 1).

One third of patients with heritable ADAMTS13 deficiency have stroke and mostly relapse if untreated, and half of them suffer from neurologic sequelae [6]. Plasma prophylaxis has not been demonstrated to prevent the end-stage renal failure in childhood [7]. The 20 years' prophylaxis controlled TTP bouts without apparent inhibitors, but did not prevent the development of some renal impairment. Our patients shared the genotype, severity, and treatment response [2,8]. Pt-3 had symptomatic urine at each infusion compared with two others. Anti-thrombotic effects require at least 5% of plasma ADAMTS13 activity [9]. Our protocol might insufficiently replace the activity to protect from chronic kidney injury. Nevertheless, the early prophylaxis in Pt-1 might sustain higher GFR. Long-term renal prophylaxis using ADAMTS13 products may overcome the limit of plasmatherapy, as in the joint prophylaxis in hemophiliacs [10].

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Abbreviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type 1, motifs 13; eGFR, estimated glomerular filtration rate; HCV, hepatitis C virus; FFP, fresh-frozen plasma; TTP, thrombotic thrombocytopenic purpura

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TABLE I. Long-Term Fresh-Frozen Plasma Prophylaxis and the Renal Outcomes of Patients With Heritable ADAMTS13 Deficiency

		ADAMTS13			Plasma prophylaxis				Renal function at present replacement				Urinalysis		
Pt	Sex	Activity (%)	Mutations derived from father and mother	Age at clinical diagnosis	Age at start (years)	Dose (ml/kg)	Interval (days)	Adverse events	Age at study (years)	Cr (mg/dl)	Cystatin C (mg/L)	eGFR (ml/min/1.73m^2)	Urine P/Cr (g/gCr)	RBC/Hb	Casts
1	f	<0.5	p.C754Afs, p.C754Afs	16 months	2	14.7	14	HCV, anaphylaxis	22	0.72	0.63	85	0.18	No	No
2	f	<0.5	p.W1081X, p.R193W	7 years	12	5.3	21	HCV	29	0.71	0.86	73	0.07	No	No
3	f	<0.5	p.Y1074Afs, p.Y1074Afs	1 month	11	7.1	18	No	32	1.22	1.70	43	0.66	Positive	Granular

Patient (Pt)-1, Pt-2, and Pt-3 correspond to U3, V3, and T4 in the reported Japanese cohort of Ref. [2]. The first diagnosis and treatment of Pt-3 was also reported in Ref. [4]. Pt-1 was born to healthy parents who were first cousins. Pt-2 and Pt-3 had unrelated healthy parents. Cr, creatinine; HCV, hepatitis C virus; eGFR, estimated glomerular filtration ratio; Urine P/Cr, urine protein to creatinine ratio, Normal range; creatinine: 0.40–0.70 mg/dl, cystatin C (a biomarker for GFR): 0.53–0.83 mg/L, eGFR: >90 ml/min/1.73 m^2, Urine P/Cr: <0.15 g/gCr.

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ADAMTS13 activity and genetic mutations in Japan

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Keywords
ADAMTS13, genetic mutation, thrombotic thrombocytopenic purpura, TTP

Summary
Thrombotic thrombocytopenic purpura (TTP), a life threatening disease, can be induced by congenital or acquired deficiency of plasma metalloprotease ADAMTS13. Since the publication of the first genetic analysis in patients with congenital ADAMTS13 deficiency in 2001, more than 100 genetic defects in the ADAMTS13 gene have been reported worldwide. Genetic analysis in patients with ADAMTS13 deficiency has greatly contributed to the understanding of the etiology of TTP. A rapid and quantitative assay method for the plasma ADAMTS13 activity was developed recently in 2005 and opened a new area of TTP research – namely genetic research using a general population to evaluate age and gender differences of ADAMTS13 activity as well as phenotype – genotype correlations of genetic polymorphisms and estimation of a homozygote or a compound heterozygote ADAMTS13 deficiencies. The Japanese general population study included 3616 individuals with an age between 30 – 80 years confirming other studies that while ADAMTS13 activity decreased with age, VWF antigen increased and VWF antigen levels are lowest in blood group O indviduals, whereas ADAMTS13 activity levels were not associated with the ABO blood group. 25 polymorphisms with a minor allele frequency of more than 0.01 were found, among them 6 missense mutations and 19 synonymous mutations, except P475S missense polymorphisms that was only idenitified in an East Asian population, characterized by reduced ADAMTS13 activity. Prevalence of congenital ADAMTS13 deficiency in the Japanese population was estimated about one individual in 1.1 x 10^6 to be homozygote or compound heterozygote for ADAMTS13 deficiency. So far more than 40 mutations in Japanese congenital TTP patients were found, but R193W, Q449*, C754Afs*24 (c.2259delA) and C908Y were identified in more than four patients suggesting the precipitaion of these mutations in the Japanese population.

Schlüsselwörter
ADAMTS13, Mutation, thrombotisch-thrombozytopenische Purpura, TTP

Zusammenfassung
Die thrombotisch-thrombozytopenische Purpura (TTP), eine lebensbedrohliche Erkrankung, kann durch kongenitalen oder erworbenen Mangel an Metalloprotease ADAMTS13 im Plasma ausgelöst werden. Seit 2001 die erste genetische Analyse bei Patienten mit kongenitalem ADAMTS13-Mangel publiziert wurde, sind weltweit mehr als 100 Gendefekte im ADAMTS13-Gen beschrieben worden. Die Genanalyse bei Patienten mit ADAMTS13-Mangel hat viel zum Verständnis der Ätiologie von TTP beigetragen. Ein schnelles und quantitatives Testverfahren wurde 2005 für die Aktivität von ADAMTS13 im Plasma entwickelt und damit ein neues TTP-Forschungsgebiet begründet – und zwar die genetische Erforschung alters- und geschlechtsbedingter Unterschiede bei der ADAMTS13-Aktivität in der Allgemeinbevölkerung, von Zusammenhängen zwischen Phäno- und Genotyp bei genetischen Polymorphismen sowie der Bewertung des homozygoten bzw. kombinierten heterozygoten ADAMTS13-Mangels. In der japanischen Bevölkerungsstudie an 3616 Personen im Alter von 30 bis 80 Jahren wurden die Ergebnisse anderer Studien bestätigt, dass zwar die ADAMTS13-Aktivität mit dem Alter abnimmt, das VWF-Antigen jedoch ansteigt, und dass die Konzentration des VWF-Antigens bei Personen mit Blutgruppe 0 am niedrigsten ist, während das ADAMTS13-Aktivitätsniveau keinen Zusammenhang mit der Blutgruppe AB0 aufwies. Es wurden 25 Polymorphismen mit einer Allelfrequenz über 0,01 gefunden, darunter 6 Missense-Mutationen und 19 synonyme Mutationen, außer dem P475S-Missense-Polymorphismus, der ausschließlich in einer ostasiatischen Population identifiziert wurde und durch reduzierte ADAMTS13-Aktivität gekennzeichnet ist. Bei der Prävalenz des kongenitalen ADAMTS13-Mangels in der japanischen Bevölkerung schätzt man, dass 1 von 1,1 x 10^6 homozygot oder kombiniert heterozygot für einen ADAMTS13-Mangel ist. Bislang wurden mehr als 40 Mutationen bei japanischen Patienten mit kongenitaler TTP entdeckt; jedoch fand man R193W, Q449*, C754Afs*24 (c.2259delA) und C908Y bei mehr als vier Patienten und nimmt daher an, dass diese Mutationen gehäuft in der japanischen Bevölkerung vorkommen.

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VWF cleavage by ADAMTS13

A disintegrin-like and metalloprotease with thrombospondin type 1 motif-13 (ADAMTS13) is a plasma metalloprotease that cleaves a specific Tyr-Met bond in the A2 domain of von Willebrand factor (VWF). The gene, which consists of 29 exons, is located on chromosome 9q34.2, and is only 129 kb distant from the ABO blood group gene.

ADAMTS13 is 1427 amino acid residues in length and consists of a

- signal peptide, a propeptide,
- reprolysin-like metalloprotease domain,
- disintegrin-like domain,
- thrombospondin type 1-like domain,
- cysteine-rich domain,
- spacer domain,
- seven additional thrombospondin type 1-like domains, and
- two CUB domains (1–3).

ADAMTS13 is mainly synthesized in stellate cells in the liver and secreted into plasma (4, 5). In humans, the plasma concentration of ADAMTS13 (6, 7) ranges from

- 0.5 to 1.0 μg/ml in Japanese and
- 0.74 to 1.42 μg/ml in Austrians.

The molecular weight of plasma ADAMTS13 was estimated to be 150 kDa by SDS-polyacrylamide gel electrophoresis (6). Thus, an ADAMTS13 plasma concentration of about 1 μg/ml is equivalent to about 6.7 nmol/l, which is less than the human plasma concentration of factor VII (10 nmol/l). Approximately 3% of ADAMTS13 in human plasma is bound to plasma VWF through its C-terminal domains (8, 9). Some mouse strains have a C-terminally truncated ADAMTS13 mutant that is probably unable to bind to VWF. Mice having a C-terminally truncated ADAMTS13 mutant show less antithrombotic activity under certain pathophysiological condition in vivo (10). Therefore, the ADAMTS13-VWF complex in plasma is functionally important for the antithrombotic activity.

The plasma half-lives of ADAMTS13 were determined as 3.3 and 2.1 days in patients with congenital ADAMTS13 deficiency following the last plasma exchange (11). The half-lives of 3.3 and 2.1 days represent the lowest known clearance rates of proteases in circulating human plasma. Plasma ADAMTS13 can bind to Lys-plasminogen in vitro but the physiological significance of the resulting compound is unknown (12).

VWF, a plasma glycoprotein of 2050 amino acid residues in length, is a large multimeric plasma glycoprotein that mediates platelet adhesion at sites of vascular injury.

VWF mediates initial platelet adhesion to the injured vessel wall by forming a bridge between subendothelial collagen and platelets in circulation (13). VWF is synthesized primarily in endothelial cells and secreted into plasma as an ultra-large (UL-VWF) multimer (>20000 kDa), some of which remains attached to the endothelial surface.

The UL-VWF multimer possesses a strong platelet aggregation ability and can spontaneously bind and aggregate platelets to generate widespread microthrombi in circulation, leading to a life-threatening disease, thrombotic thrombocytopenic purpura (TTP) (14–16). Under normal conditions, the UL-VWF multimer can be depolymerized by ADAMTS13. Congenital or acquired deficiency of ADAMTS13 leaves the UL-VWF multimer intact, leading to TTP. Platelets and coagulation factor VIII can accelerate the VWF cleavage by ADAMTS13 (17, 18). The plasma concentration of VWF is about 10 μg/ml, which is 10-fold higher than the concentration of ADAMTS13.

VWF has a discrete domain structure that can be clearly observed by an electron microscope (19). In the central region of the polypeptide chain, VWF has three successive A domains, A1-A3, with a high sequence identity. Each A domain is about 90 amino acid residues in length. The A1 domain has a binding region for platelet glycoprotein Ib and the A3 domain has a binding site for subendothelial collagen (13). The A1 and A3 domains have a disulfide bond that forms a link between the N- and C-terminal regions. The A2 domain, however, does not have a corresponding disulfide bond and instead has a vicinal disulfide bond in the C-terminal region. The crystal structure of the A2 domain suggested that the C-terminal half of the domain can be easily unfolded so that the scissile bond, Tyr1605-Met1606, for ADAMTS13 is exposed (20). As to how ADAMTS13 specifically cleaves this unique single peptide bond in the A2 domain of VWF, new information has been accumulating.

The basic concept for the cleavage is that the scissile bond in the A2 domain is cryptic and sequestered and the shear stress partially unfolds the A2 domain, resulting in exposure of this bond (21, 22).

ADAMTS13 is constitutively active in plasma and the exposed scissile bond in VWF can be easily cleaved by ADAMTS13. Thus, the shear stress-dependent substrate-binding mechanism of ADAMTS13 in vivo is very unique.

VWF73 as a minimal substrate for ADAMTS13

Although identification of the proteolytic cleavage site, the Tyr1605-Met1606 bond, in VWF was reported more than two decades ago (23), a plasma assay for the ADAMTS13 activity was a laborious and time-consuming work (24, 25). In order to develop a fast, quantitative, and synthetic substrate for the VWF-cleaving activity of ADAMTS13, a minimal sequence specifically recognized and cleaved by ADAMTS13 should be determined in VWF.

To identify the region in VWF required for ADAMTS13 cleavage, we expressed a series of deletion mutants of the A2 domain and found that a 73-amino-acid fragment from Asp1596 to Arg1668 was essential for the cleavage of the Tyr1605-Met1606 bond by ADAMTS13 (26). We named this fragment VWF73. A 64-amino-acid fragment from Asp1596 to Arg1659 was not efficiently cleaved by ADAMTS13. We determined the solution structure of the ^{1}H and ^{15}N double-labeled substrates VWF73 and VWF64, each of which included a C-terminal 6xHis tag, by nuclear magnetic resonance. The results

indicated an extended structure for both peptides, suggesting an induced-fit substrate recognition mechanism (27).

VWF73 has all the characteristics of an ADAMTS13 substrate. We developed a chemically modified VWF73 for use in a fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity. This substrate, FRETS-VWF73, quantitatively and reproducibly yielded the activity of plasma ADAMTS13 within one hour (24), which constitutes a remarkable improvement in rapidity and accuracy over the previous assays (28, 29). Using this assay, we were able to show that patients with congenital TTP exhibited severely decreased (<5% of the reference value) or undetectable ADAMTS13 activities. A slightly modified method using FRETS-VWF73 can quantitatively detect ADAMTS13 activity of less than 5% of the reference value (30).

An enzyme immunoassay of ADAMTS13 activity using a monoclonal antibody that specifically recognizes Tyr1605, the C-terminal residue of the cleaved A2 domain, has been developed and a chromogenic ADAMTS13-act-ELISA using a glutathione-conjugated VWF73 peptide as the substrate has also been reported (25).

These assays are utilized for the clinical diagnosis of TTP.

ADAMTS13 activity in the Japanese population

The Suita Study is an epidemiological study consisting of Japanese residents between the ages of 30 and 79 years who were randomly selected from the municipality population registry and stratified into groups by sex and age in 10-year increments. We have used FRETS-VWF73 to measure the plasma ADAMTS13 activity in 3616 individuals from this general population with age ranged from 30 to 80 years.

- When the mean of all plasma ADAMTS13 activity values was set at 100%, the mean activity of men (93 ± 24%, n = 1687) was significantly lower than that of women (106 ± 27%, n = 1929) (31).
- The plasma ADAMTS13 activity tended to decrease with age, especially after age 60, in both men and women. The mean ADAMTS13 activity value was
  - 110% for subjects in their 40s,
  - 109% for those in their 50s,
  - 101% for the 60s,
  - 93% for the 70s, and
  - 85% for the 80s.

We also measured the plasma VWF antigen level in this population. The VWF antigen level increased with age, as reported previously. Because of the combined effects of the increase in VWF antigen level and the decrease in ADAMTS13 activity, the ratio of VWF antigen-to-ADAMTS13 activity was dramatically increased with age (31). This may partly explain the prothrombotic state of elderly men and women. As the FRETS-VWF73 assay itself was not affected by VWF concentration in plasma samples (0–160 ug/ml) (31), the reduced ADAMTS13 activity in the plasma of elderly subjects was not considered to be due to the assay-dependent artifactual phenomenon. In fact, when age-adjusted VWF antigen level was compared among quartiles of ADAMTS13 activity in the population, no significant association between VWF antigen and ADAMTS13 activity levels was observed in men or in women.

The AB0 blood group is a well-known genetic determinant for plasma VWF antigen levels:

Individuals with blood group 0 have a lower VWF level than those with non-0 groups (32).

The AB0 blood group gene is located approximately 129 kb from the ADAMTS13 gene, and this may suggest a possible correlation between the two genes. In our population, the individuals with blood group 0 exhibited a significantly lower VWF antigen level than those with non-0 groups, as shown in previous studies. In contrast, the plasma ADAMTS13 activity was not associated with the AB0 blood group (31). This was consistent with the observation that ADAMTS13 antigen levels were not associated with AB0 blood group in 387 male Dutch individuals (33). The results are also consistent with the fact that VWF (34), but not ADAMTS13 (35), contains AB0 blood group-related N-linked oligosaccharides.

Control plasma for ADAMTS13 assay

The level of ADAMTS13 activity in the general population varied widely, ranging from approximately 40% to 240% of the normal level (▶ Fig. 1). In general, the plasma ADAMTS13 activity is expressed as a percentage of the activity in commercially available or locally prepared, pooled normal plasma (control plasma). Therefore, if there is a wide range of ADAMTS13 activity among the control plasma samples be-

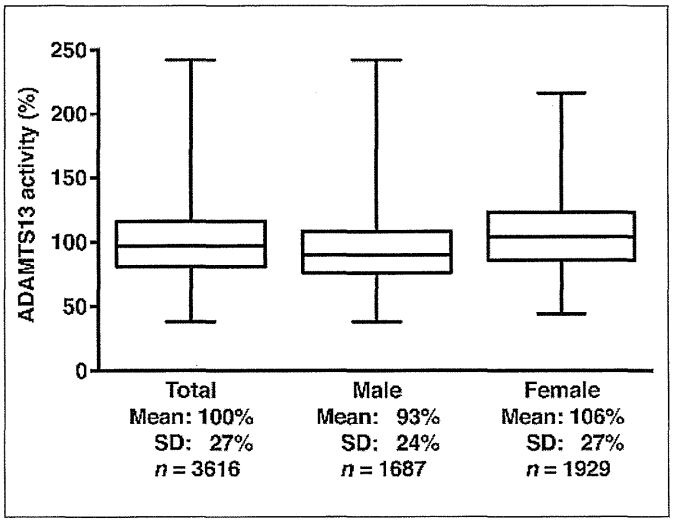

Fig. 1 Plasma ADAMTS13 activity in a Japanese population. Box-and-whisker plots of the plasma ADAMTS13 activities in men, women, and all subjects are shown. The mean of all values in total subjects was set at 100%. The top and bottom of the box are the 75th and 25th percentiles. The whiskers go down to the smallest value and up to the largest.

fore pooling, this can create a serious problem for ADAMTS13 measurement. It is easy to assume that the control plasma samples prepared from a relatively small number of individuals would show large deviation. In order to estimate the ideal number of individuals for the preparation of the control plasma, we randomly selected the ADAMTS13 activity values from 10 individuals (5 men and 5 women) in the general population cohort consisting of 3616 individuals, and repeated this selection 10 times to obtain the mean ± 2 standard deviation. The results of the 10-times repeated selection showed that 80% was the value of the mean – 2 standard deviations and 125% was the value of the mean + 2 standard deviations. These results indicated that the control plasma randomly prepared from 10 individuals represented a wide variation of activity and thus was not suitable for use as a control.

When we selected the activities of 20 individuals (10 men and 10 women), the activities of the mean + and – 2 standard deviations were narrowed down to 89% and 113%, respectively, which might have been sufficient for diagnostic purposes. When we selected 40 individuals or 100 individuals, the activity ranges were reasonably narrowed and were useful for the rigorous analysis of the ADAMTS13 activity (40 individuals: mean ± 2 standard deviations, 91–108%; 100 individuals: mean ± 2 standard deviations, 94–104%). As described, sex and age influence plasma ADAMTS13 activity. Therefore, the control plasma for plasma ADAMTS13 activity can be prepared from at least 20 individual plasma samples in consideration of age and sex.

Currently, a new project, "*Development of the WHO 1st International Standard for ADAMTS13 in Plasma*" led by the VWF Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis has been initiated by Dr. Johanna Kremer Hovinga.

Genotype-phenotype correlation of polymorphisms

The ADAMTS13 gene contains several genetic missense polymorphisms (3, 36, 37) some of which may influence the VWF cleaving activity. Since low plasma ADAMTS13 and high VWF levels are related to ischaemic stroke and myocardial infarction (38–40), missense polymorphisms in the ADAMTS13 gene could be important. Twenty missense polymorphisms of the ADAMTS13 gene have been listed (37), and some of their possible structural defects have been examined in silico (41).

To identify genetic polymorphisms in the Japanese population, we sequenced the ADAMTS13 gene in 346 individuals and identified 25 polymorphisms with a minor allele frequency of > 0.01 (42):

- 6 were missense polymorphisms and
- 19 were synonymous mutations.

We further genotyped six missense polymorphisms in a large Japanese cohort consisting of 3616 individuals whose plasma ADAMTS13 activities had been measured. We found that the minor allele frequencies were

- 0.192 for Q448E (c.1342C>G),
- 0.05 for P475S (c.1423C>T),
- 0.048 for S903L (c.2708C>T),
- 0.027 for T339R (c.1016C>G),
- 0.027 for P618A (c.1852C>G) and
- 0.022 for G1181R (c.3541G>A).

The T339R and P618A polymorphisms were in absolute linkage disequilibrium. When we examined the association of these polymorphisms with plasma ADAMTS13 activity, the ADAMTS13 activity of Q448E heterozygotes (QE) and minor allele homozygotes (EE) was significantly higher than that of major allele homozygotes (QQ):

- QQ: 97.6% ± 25.9%;
- QE: 104.2% ± 27.4%;
- EE: 105.7% ± 27.5%.

In contrast, the ADAMTS13 activity of P475S heterozygotes (PS) and minor allele homozygotes (SS) was significantly lower than that of major allele homozygotes (PP):

- PP: 101.4% ± 26.6%;
- PS: 87.2% ± 23.3%;
- SS: 73.3% ± 20.3% (42).

Four other missense polymorphisms did not affect the ADAMTS13 activity.

Tab. 1 Non-synonymous mutations identified in four segregated groups with different ranges of ADAMTS13 activity

group (average activity)	mutation	predicted damage	references
maximum (183%)	L19F	benign	newly identified
	R268Q		
median (97.6%)	Q723K		
	N1321S		
2nd minimum (53.1%)	I380T		
	Y1074Afs*46	–	causative for congenital ADAMTS13 deficiency (46)
	R1274C	possibly damaging	newly identified
minimum (47.1%)	F324L	probably damaging	
	F418L		
	I673F	possibly damaging	causative for congenital ADAMTS13 deficiency (47)
	Q773*	–	newly identified
	Y1074Afs*46	–	causative for congenital ADAMTS13 deficiency (46)
	R1095Q	probably damaging	newly identified

*stop codon

As described, the AB0 blood group gene is located near the ADAMTS13 gene. T339R and P618A were associated with the blood group A allele and P475S and S903L tended to be associated with the blood group 0 allele in our study (42).

The P475S missense polymorphism is ethnic specific, having only been identified in an East Asian population. The reduced plasma ADAMTS13 activity in individuals with the P475S mutation is consistent with the finding that the recombinant ADAMTS13-P475S mutant showed approximately 70% of the activity of the wild-type ADAMTS13 (43). To further elucidate the molecular basis of the reduced activity of the ADAMTS13-P475S mutant, we recently determined the enzymatic parameters of ADAMTS13-MDTCS (residues 75–685) and MDTCS-P475S and solved the crystal structure of the P475S mutant of the ADAMTS13-DTCS domain (44). MDTCS-P475S exhibited a reaction rate similar to that of wild-type MDTCS but showed twofold lower affinity for FRETS-VWF73, indicating that Pro475 is involved in formation of the substrate-binding exosite. The crystal structures showed that the conformation of the P475S-containing loop was significantly different between the mutant and the wild-type. This explains the higher susceptibility of the enzymatic activity of MDTCS-P475S to environmental conditions such as denaturants and high temperature. MDTCS-P475S can moderately cleave shear-treated VWF.

Incompatible evidences between in vivo and in vitro studies are accumulating on one of the missense polymorphisms, P618A. PolyPhen-2, an in silico tool which predicts the possible impact of an amino acid substitution on the structure and function of a human protein, predicted P618A as a damaging mutation. The crystal structure of the S domain of ADAMTS13 showed that Pro618 adopted the *cis* conformation (41), and the substitution of Pro618 with Ala, which cannot adopt the *cis* conformation, may cause structural distortion. Indeed, a transient expression study of the ADAMTS13-P618A mutant showed lower levels of activity and antigen in the conditioned media of HEK293 cells (45). However, as described, the P618A mutation was not associated with plasma ADAMTS13 activity in the general population. This inconsistent observation should be properly addressed in the future experiments.

ADAMTS13 deficiency in a Japanese population

The ADAMTS13 activity-genotype analysis based on ~3200 individuals enabled us to estimate the frequency of congenital ADAMTS13 deficiency. We selected 128 individuals according to their plasma ADAMTS13 activity:

- 32 individuals of the "minimum" activity group (average activity, 47.1%),
- 32 individuals of the "second minimum" activity group (average activity, 53.1%),
- 32 individuals of the "median" activity group (average activity, 97.6%), and
- 32 individuals of the "maximum" activity group (average activity, 183%).

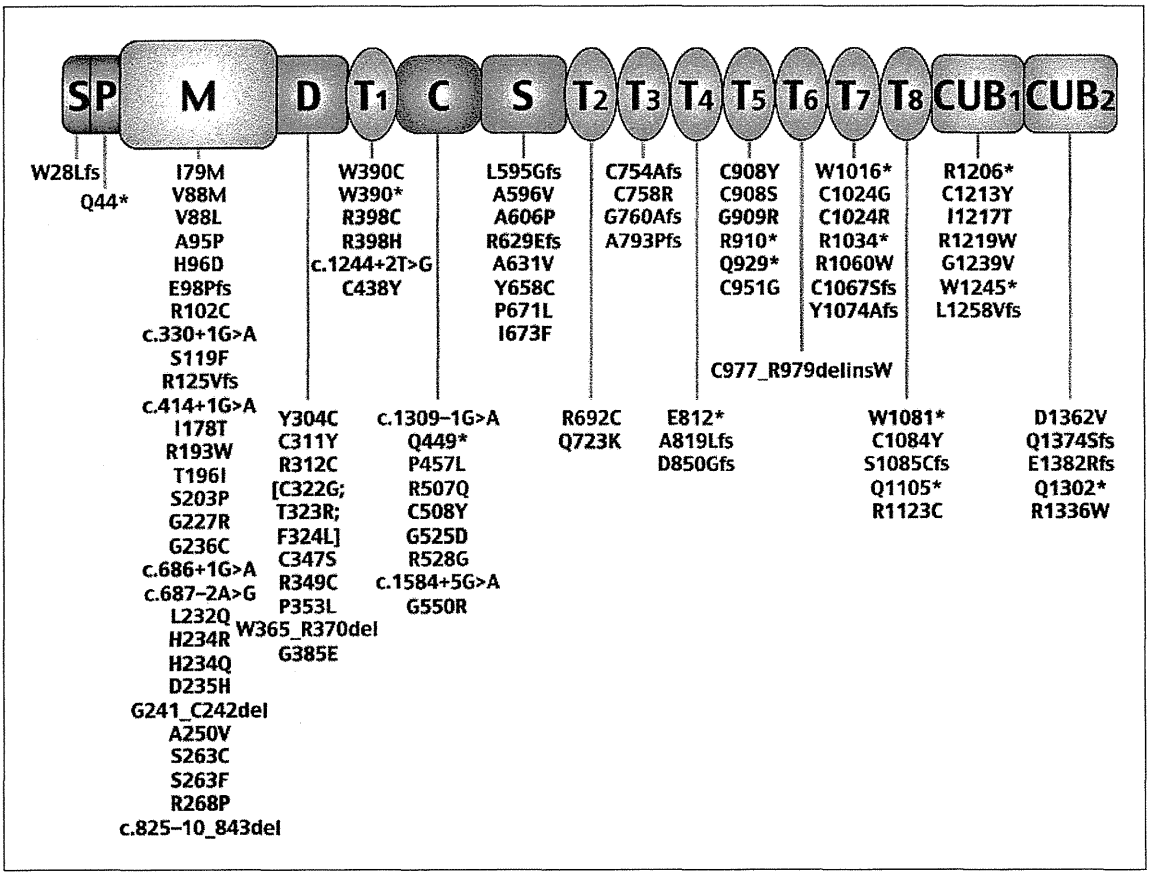

Fig. 2
ADAMTS13 gene mutations responsible for congenital TTP (*stop codons). The description of protein sequence mutation follows the recommendation of the Human Genome Variation Society (www.hgvs.org/mutnomen/recs-prot.html). Mutations in red were identified in Japanese patients.

Sequence analysis of the ADAMTS13 gene in these individuals showed that 14 individuals had rare non-synonymous mutations: seven individuals in the minimum activity group, three individuals in the second minimum activity group, two individuals in the median activity group, and two individuals in the maximum activity group (▶Tab. 1). In particular, three of the subjects had causative mutations for congenital ADAMTS13 deficiency, Y1074Afs*46 (46) and I673F (47). These data indicated that 2 of every 32 individuals had a mutation that does not cause a functional defect of ADAMTS13. Therefore, it would be a reasonable assumption that five individuals in the minimum activity group and one individual in the second minimum activity group would be heterozygotes carrying a mutation with a functional defect. If this assumption is valid, 6 out of 3200 individuals would be heterozygotes for ADAMTS13 deficiency. This estimation suggested that ~ 1 individual in 1.1×10^6 should be a homozygote or a compound heterozygote for ADAMTS13 deficiency. If a part of homozygous/compound heterozygous mutation carriers would die during the neonatal period, the prevalence in the surviving population may be lowered. It has been reported that the E1382Rfs*6 mutation (the E1382R frameshift mutation giving rise to the stop codon at six amino acid residues thereafter) due to the 4143insA mutation is frequent among patients with congenital ADAMTS13 deficiency in Northern and Central European countries (48). The estimation of the prevalence of patients with congenital ADAMTS13 deficiency may be biased due to insufficient sample sizes, ethnicity, lethality, and other factors.

ADAMTS13 mutations in congenital TTP

Since the publication of the first genetic analysis in patients with Upshaw-Schulman syndrome in 2001 (3), more than 100 genetic defects in the ADAMTS13 gene have been reported worldwide (36, 37, 49). The genetic variants that lead to TTP are very broadly distributed, occurring everywhere from the N-terminal signal peptide to the C-terminal CUB domain. The missense mutations are most frequent (about 60%), but other non-synonymous mutations such as frameshift mutations (small deletions or insertions), nonsense mutations, abnormal splicing, and insertions/deletions, are also detected (▶Fig. 2).

> We have so far identified more than 40 genetic mutations in Japanese patients with congenital TTP.

Most of the mutations were found in a single patient, but four mutations, i.e., R193W, Q449*, C754Afs*24 (c.2259delA), and C908Y, were identified in more than four patients, suggesting the accumulation of these mutations in a Japanese population (46).

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Conflict of interest

The National Cererbral and Cardiovascular Center where TM and KK (inventors) belong has an awarded patent on the use of reagent, FRETS-VWF73. MM is al clinical advisory board for Alexion Pharmaceuticals, and has a patent on the use of chromogenic ADAMTS13 activity assay using the nonoclonal antibody, which specifically recognizes TYR1605 within VWF-A2 domain, exposed by ADAMTS13 cleavage. YF is a clinical advisory board for Baxter Bioscience and for Alexion Pharmaceuticals, and has a patent on the use of chromogenic ADAMTS13 activity assay using the nonoclonal antibody, which specifically recognizes TYR1605 within VWF-A2 domain, exposed by ADAMTS13 cleavage.

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