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ORIGINAL PAPER

ADAMTS13 phenotype in plasma from normal individuals and patients with thrombotic thrombocytopenic purpura

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Abstract The activity of ADAMTS13, the von Willebrand factor cleaving protease, is deficient in patients with thrombotic thrombocytopenic purpura (TTP). In the present study, the phenotype of ADAMTS13 in TTP and in normal plasma was demonstrated by immunoblotting. Normal plasma (n=20) revealed a single band at 190 kD under reducing conditions using a polyclonal antibody, and a single band at 150 kD under non-reducing conditions using a monoclonal antibody. ADAMTS13 was not detected in the plasma from patients with congenital TTP (n=5) by either antibody, whereas patients with acquired TTP (n=2) presented the normal phenotype. Following immunoadsorption of immunoglobulins, the ADAMTS13 band was removed from the plasma of the patients with acquired TTP, but not from that of normal individuals. This indicates that ADAMTS13 is complexed with immunoglobulin in these patients. The lack of ADAMTS13 expression in the plasma from patients with hereditary TTP may indicate defective synthesis, impaired cellular secretion, or enhanced degradation in the circulation. This study differentiated between normal and TTP plasma, as well as between congenital and acquired TTP. This method may, therefore, be used as a complement in the diagnosis of TTP.

Keywords ADAMTS13 · von Willebrand factor · Thrombotic thrombocytopenic purpura · Immunoblotting · Plasma · von Willebrand factor cleaving protease

Abbreviations

ADAMTS13	a disintegrin-like and metalloprotease with thrombospondin-type-1 motifs, 13
ELISA	enzyme-linked immunosorbent assay
HUS	hemolytic uremic syndrome

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TMA	thrombotic microangiopathy
TTP	thrombotic thrombocytopenic purpura
ULVWF	ultra-large von Willebrand factor
VWF	von Willebrand factor

Introduction

Von Willebrand factor (VWF) is a glycoprotein that plays a key role in the primary hemostatic process by inducing platelet adhesion and aggregation at sites of vascular injury under conditions of high shear stress. The main source of circulating VWF is the endothelium, from which it is secreted in the form of ultra-large multimers (ULVWF) [53]. ULVWF multimers are biologically very active [2, 31] and, upon release, undergo processing into smaller multimers in normal individuals. This occurs on the surface of endothelial cells [10]. VWF defects may potentially lead to both bleeding and thrombotic disorders: defective VWF secretion, intravascular clearance, multimer assembly, or increased proteolytic degradation may lead to different types of von Willebrand disease. On the other hand, dysfunctional VWF proteolysis may lead to the thrombotic disorder thrombotic thrombocytopenic purpura (TTP) [40].

TTP is a thrombotic microangiopathy (TMA) characterized by microangiopathic hemolytic anemia, thrombocytopenia, fever, neurological and renal manifestations. Chronic recurrent TTP has been associated with the presence of ULVWF in the plasma [30]. ULVWF multimers are capable of inducing increased platelet retention in children with TTP [21]. These observations, along with the finding of VWF and platelet-rich (but fibrin-poor) thrombi in the microcirculation of the heart, brain, kidneys, liver, spleen, and adrenals in TTP patients [3], led to the conclusion that ULVWF multimers are responsible for the disseminated platelet thrombi occurring in TTP and that their degradation to smaller VWF multimers is impaired due to the deficiency of a VWF-cleaving protease [15].

Recently, the VWF-cleaving protease was purified [12, 13, 16, 50] and the encoding gene sequenced, linking the protease to the ADAMTS (a disintegrin-like and metalloprotease with thrombospondin-type-1 motif) family of metalloproteases [27]. The protease, named ADAMTS13, cleaves VWF at the 1605Tyr-1606Met peptide bond in the A2 domain, yielding the 140-kD and 176-kD VWF fragments present in normal plasma [13, 50]. Cleavage is made possible by a conformational change in VWF due to shear stress in the circulation, which exposes the cleavage site, making it susceptible to proteolysis [55]. ADAMTS13 activity is severely deficient (<5% of normal plasma activity) in TTP patients [6], either due to a mutation in the *ADAMTS13* gene in the congenital form of TTP or due to auto-antibodies in the acquired form [14, 27, 52]. Autosomal recessive hereditary TTP (also termed the Upshaw-Schulman syndrome) typically presents during the neonatal period or early childhood (<10 years of age), but may also manifest during adolescence and adulthood. Recurrent TTP episodes may occur as often as every third week. TTP recurrences are associated with cerebral vascular accidents in approximately 30% of cases, and these episodes may lead to neurological complications. Renal manifestations may be mild or may result in acute renal failure due to hemoglobinuria and TMA. About 20% of patients progress to end-stage renal failure [28].

Hemolytic uremic syndrome (HUS) is a similar microangiopathic disorder characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure [5]. Two forms of HUS have been described: D+ or typical (diarrhea-associated) HUS and D- or atypical (non-diarrhea-associated) HUS. D+ HUS occurs after infection with Shiga-like toxin producing bacteria, typically, enterohemorrhagic *Escherichia coli*. The patients are usually children presenting with abrupt onset of diarrhea, followed by the development of HUS 2–10 days later. A prothrombotic state precedes the acute renal failure [8], but the pathogenetic mechanism is, as yet, unclear. It is assumed that bacterial virulence factors gain access to the circulation, circulate on blood cells, activate platelets, and reach the kidney, where the endothelium is injured [36, 47]. D- HUS is associated with mutations in certain complement regulatory factors, such as factor H, factor I, and membrane co-factor protein (CD46). The mutations lead to activation of the complement system on host endothelial cells [29, 58]. The resulting vascular damage may lead to the formation of thrombotic lesions in the kidneys.

Although HUS patients are, typically, young children with a history of diarrhea and acute renal failure, the clinical manifestations of HUS and TTP often overlap, making differentiation between the two syndromes based solely on clinical presentation difficult. ADAMTS13 antigen levels can differentiate between HUS and TTP, as they are severely deficient in patients with congenital TTP and normal to moderately reduced in HUS [51]. Assays for ADAMTS13 activity can, therefore, differentiate between TTP (congenital and acquired) and HUS [26, 48].

Several ADAMTS13 assays are available today based on antigen detection and activity [15, 17, 25, 38], showing the presence of the protease (by enzyme-linked immunosorbent assay, ELISA) and its bioactivity in normal plasma and the lack of protease and activity in the plasma from patients with congenital TTP. These assays have also shown that patients with acquired TTP have auto-antibodies that neutralize the activity of ADAMTS13. The present study utilized a different method, immunoblotting, and two anti-ADAMTS13 antibodies against specific domains, to inves-

tigate the presence of ADAMTS13 antigen in normal plasma, TTP plasma (congenital and acquired), and in heterozygous carriers of ADAMTS13 mutations, demonstrating the presence of ADAMTS13 and its size in normal plasma, the lack thereof in congenital TTP, and autoantibody-bound protease in acquired TTP.

Materials and methods

Subjects

Citrated plasma was available from patients with congenital (n=5) and acquired (n=2) TTP. The patient data are presented in Table 1. The ADAMTS13 activity level was assayed as previously described [15, 17].

The study also included the parents of patients 1–4. The parents of patients 1 and 2 are both heterozygous for the 4143insA mutation, and have protease activity levels of 20% (mother) and 50% (father), as assayed by the VWF multimeric structure analysis [15]. The parents of patient 3 are heterozygous for the P353L (mother) and P457L (father) mutations, and both have 50% ADAMTS13 activity. The parents of patient 4 are heterozygous for P671L (mother) and 4143insA (father), and have 50% ADAMTS13 activity. All parents are clinically unaffected.

Plasma samples from 20 healthy adult volunteers were used as controls. The study was conducted with the approval of the ethics committee of Lund University and the plasma samples were collected with the informed consent of the patients, their parents, and the controls.

Table 1 Clinical and laboratory data regarding thrombotic thrombocytopenic purpura (TTP) patients

Patient no.	Sex	Age at debut	Age at sampling (years)	Current age (years)	Symptoms during episodes	No. of episodes	ADAMTS13 activity level	ADAMTS13 mutation	ADAMTS13 inhibitor	Reference
1[a]	M	2 d	16	19	Jaundice, hemolytic anemia, thrombocytopenia, macroscopic hematuria, pathological urinalysis, fever, neurological symptoms, elevated serum creatinine	>5	<5%	4143insA[bc]	None	[4, 20, 43]
2[a]	M	5.3 y	17	18	Jaundice, hemolytic anemia, thrombocytopenia, fever, neurological symptoms, pathological urinalysis	>5	<5%	4143insA[bc]	None	[4, 20, 43]
3	F	20 m	15	23	Hemolytic anemia, thrombocytopenia, hematuria, epileptic attacks, slightly elevated serum creatinine	>5	<5%	P353L[d], P457L[e]	None	[4]
4	M	3 y	7	9	Hemolytic anemia, thrombocytopenia, purpura, pathological urinalysis	4	<5%	P671L[f], 4143insA	None	[43]
5	M	2 d	39	39	Jaundice, hemolytic anemia, petechiae, thrombocytopenia, transitory neurological deficits and aphasia, elevated creatinine	>5	<5%	4143insA[b]	None	–
6	F	54 y	70	75	Recurrent hemolytic anemia and thrombocytopenia, reduced consciousness, pathological urinalysis	>5	<5%	NA	0.5 U/ml	–
7	F	25 y	42	44	Thrombocytopenia, hemolytic anemia, elevated creatinine during viral infection and pregnancy	2	<5%	NA	0.2 U/ml	–

[a] Patients 1 and 2 are siblings
[b] Patients 1, 2, and 5 are homozygous for the ADAMTS13 mutation
[c] 4143insA leads to a mutation in the second CUB domain
[d] P353L is a mutation in the disintegrin-like domain
[e] P457L is a mutation in the cysteine-rich domain
[f] P671L is a mutation in the spacer domain
NA: not assayed

Plasma samples

At the time of sampling, the patients were treated regularly with fresh frozen plasma or Octaplas (Octapharma, Stockholm, Sweden; patients 1–5). Patient 6 was treated with plasma infusions every sixth week. Patient 7 did not receive any plasma treatment at sampling. All blood samples were obtained at least three weeks (patients 1–4 and 6) or one week (patient 5) after the last treatment. Venous blood from patients and controls was collected, and the plasma obtained as previously described [20].

Anti-ADAMTS13 antibodies

A polyclonal anti-peptide antibody was raised in New Zealand white rabbits against a unique sequence in the second CUB domain (AA1413-1427) and affinity-purified against the peptide. Antibody specificity was tested by ELISA (plates coated with the peptide) and by immunoblotting with purified plasma ADAMTS13 [50] under reducing (SDS-PAGE) and non-reducing (dot blot) conditions.

The monoclonal antibody A10 [56], directed against the disintegrin-like domain, was used to confirm the results of the polyclonal antibody. The polyclonal antibody reacted with ADAMTS13 under reducing (reduced by the addition of 2-mercaptoethanol to disrupt disulfide bonds in the protease) and non-reducing conditions, whereas the monoclonal antibody reacted with ADAMTS13 only under non-reducing conditions.

Immunoblot analysis for the detection of ADAMTS13 in plasma

The plasma samples (1:20) were subject to SDS-PAGE under reducing (for blotting with the polyclonal antibody) and non-reducing conditions (for blotting with the monoclonal antibody) [19, 58]. Purified plasma ADAMTS13 (1:100) was used as the control for the polyclonal antibody. Immunoblotting was performed with rabbit anti-ADAMTS13 IgG 1.6 μg/ml followed by goat anti-rabbit IgG HRP (DakoCytomation, Carpinteria, CA) 1:2000, or with mouse anti-ADAMTS13 IgG 0.6 μg/ml followed by goat anti-mouse IgG HRP (DakoCytomation, Carpinteria, CA) 1:2000. The signal was detected by chemiluminescence. The specificity of the signal obtained with the polyclonal antibody was tested by preincubation with a 50-fold molar surplus of blocking peptide followed by immunoblotting with the blocked antibody. The specificity of the secondary antibodies was tested by omission of the primary antibodies.

Immunoblotting with the polyclonal antibody revealed, in addition to ADAMTS13, two unspecific bands at 130 kD and 170 kD, which were identified as C3 and alpha-2-macroglobulin. These proteins were removed by incubating the plasma samples with protein A-sepharose coupled rabbit anti-C3 IgG and rabbit anti-alpha-2-macroglobulin IgG. The results obtained using the polyclonal antibody show samples from which these proteins have been removed.

In order to investigate the presence of ADAMTS13-autoantibody complexes in the plasma from patients with acquired TTP, samples were passed onto protein G-sepharose (Amersham Biosciences, Buckinghamshire, UK) prior to immunoblotting. Normal plasma (n=2) was used for comparison.

Results

Detection of ADAMTS13 in plasma samples from normal individuals

Normal plasma under reducing conditions revealed a single immunoreactive band at 190 kD when blotted against the polyclonal antibody (Fig. 1a). Purified plasma ADAMTS13 showed a similar band under the same conditions (Fig. 1a). The monoclonal antibody detected an immunoreactive band at 150 kD in normal plasma under non-reducing conditions (Fig. 1b). Preincubation of the polyclonal antibody with the blocking peptide abolished the ADAMTS13 band in normal plasma (data not shown). Immunoblots in which the primary antibodies had been omitted showed no bands (data not shown).

Detection of ADAMTS13 in plasma samples from TTP patients

The plasma samples from all patients with congenital TTP, regardless of the mutation, lacked the ADAMTS13 band (Fig. 1c). Patients 6 and 7 with acquired TTP revealed the same ADAMTS13 protein expression pattern as the controls (Fig. 1c). These results were obtained using both the polyclonal (Fig. 1c) and the monoclonal antibody (Fig. 1d). When immunoadsorption of plasma immunoglobulins was carried out prior to immunoblotting, the ADAMTS13 band remained visible in normal plasma, but was completely removed from the samples of the patients with acquired TTP (Fig. 1e), probably due to its association with the immunoglobulin inhibitor.

Detection of ADAMTS13 in the parents of the TTP patients

The parents of the TTP patients are all carriers of one *ADAMTS13* mutation and one normal allele. All parents presented a normal ADAMTS13 phenotype using both the polyclonal (data not shown) and the monoclonal antibody (Fig. 1g).

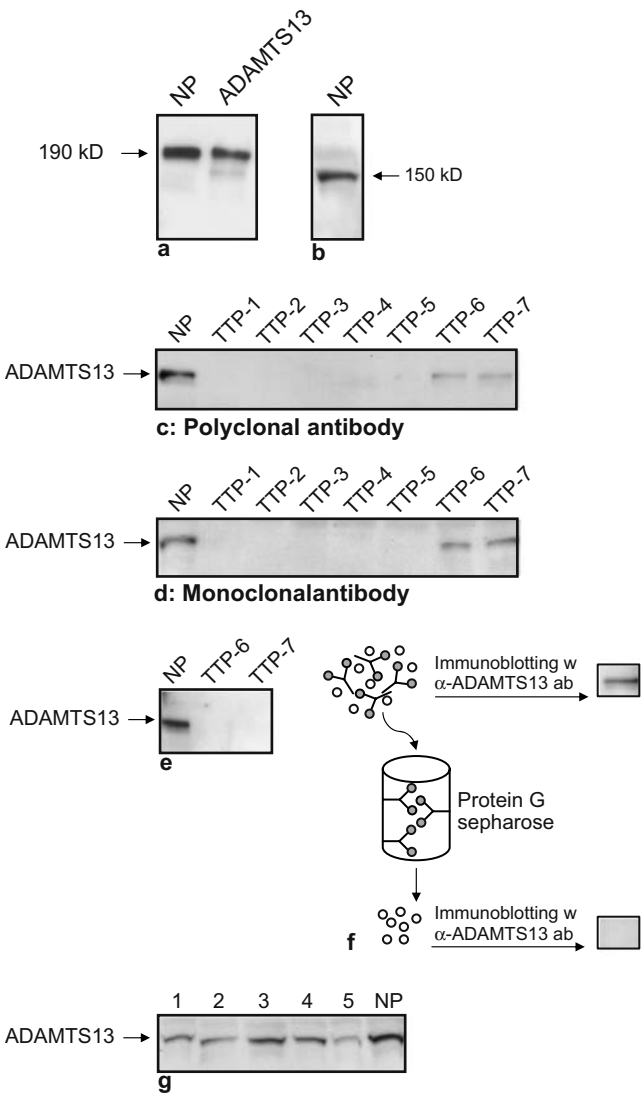

Fig. 1a–g Detection of ADAMTS13 in normal and thrombotic thrombocytopenic purpura (TTP) plasma. **a** The polyclonal antibody detected a single band at 190 kD in normal plasma (NP) under reducing conditions. Purified plasma ADAMTS13 in the right lane showed a similar band. **b** Normal plasma under non-reducing conditions revealed an immunoreactive band at 150 kD when blotted against the monoclonal antibody. **c** Immunoblotting with the polyclonal antibody revealed that patients 1–5 with congenital TTP all lacked the ADAMTS13 band, whereas patients 6 and 7 with acquired TTP presented a normal expression pattern. Normal plasma (NP) was run on the same gel for comparison. **d** These results were confirmed by the monoclonal anti-ADAMTS13 antibody. **e** Immunoblot using the polyclonal antibody. Immunoadsorption of immunoglobulins from the plasma samples of patients 6 and 7 with acquired TTP lead to the simultaneous removal of the ADAMTS13 band, indicating that ADAMTS13 is complexed with the anti-ADAMTS13 auto-antibodies. In contrast, the ADAMTS13 band remained visible in normal plasma (NP) treated similarly. **f** A schematic presentation of the mechanism by which the removal of immunoglobulins leads to the removal of the ADAMTS13 antigen from the plasma of patients with acquired TTP. The plasma sample contains ADAMTS13 (*filled circles*), auto-antibodies to ADAMTS13 (⊣), and various other plasma proteins (*open circles*). Immunoblotting of the plasma sample prior to the removal of immunoglobulins detects the presence of ADAMTS13 antigen. Passage of the plasma sample through a protein G-sepharose column leads to the binding and removal of all immunoglobulins from the sample. Since ADAMTS13 is bound to the anti-ADAMTS13 auto-antibodies, it is removed along with them. Immunoblotting of the flow-through shows no ADAMTS13 band. **g** Immunoblot with the monoclonal antibody showing a normal ADAMTS13 band in the plasma of the parents, which are all heterozygous for one mutated allele and are clinically unaffected. Lane 1: the mother of patients 1 and 2, 4143insA; lane 2: the mother of patient 3, P353L; lane 3: the father of patient 3, P457L; lane 4: the mother of patient 4, P671L; lane 5: the father of patient 4, 4143insA. Normal plasma (NP) was run on the same gel for comparison

Discussion

In the present study, we detected ADAMTS13 in plasma using a polyclonal and a monoclonal antibody. This assay was capable of distinguishing TTP patients from normal individuals, as well as differentiating between congenital and acquired TTP. Plasma from the patients with congenital TTP lacked the ADAMTS13 antigen. In contrast, the plasma of patients with acquired TTP expressed a normal ADAMTS13 phenotype.

Previous studies describing the ADAMTS13 phenotype in normal plasma by immunoblotting with other specific anti-ADAMTS13 antibodies have shown immunoreactive bands of the same molecular weight using similar conditions [33, 46]. The ADAMTS13 antigen in patients with congenital and acquired TTP has recently been shown by ELISA, demonstrating low to undetectable ADAMTS13 levels in patients with congenital TTP [11, 38] and decreased, but mostly detectable, levels in patients with acquired TTP [11, 38, 45]. In the present study, the ADAMTS13 phenotype in TTP patients is described by immunoblotting, confirming the lack of ADAMTS13 antigen in the plasma of patients with congenital TTP and the presence of circulating complexes in acquired TTP. Furthermore, we showed that heterozygous carriers of the ADAMTS13-related mutations who, thus, have reduced ADAMTS13 bioactivity have a normal phenotype.

The plasma of the patients with congenital TTP did not present the ADAMTS13 band. This may be due to altered synthesis, secretion or antigenicity, or due to increased breakdown of the protease in plasma. The fact that two antibodies directed to two different domains in ADAMTS13 were unable to detect the protease band makes altered antigenicity less likely to be the cause for the lack of the ADAMTS13 band in these patients. Previous studies have shown impaired secretion of the 4143insA (patients 1, 2, 4, and 5) [35, 42, 44] and P353L (patient 3) [42] mutants from cells, thus, indicating that the protease may accumulate intracellularly, at least in some patients with congenital TTP. This may be due to a missing

cell sorting signal, as in the case of 4143insA [44] or due to conformational changes in the protein, impairing its secretion. Similar findings regarding two other ADAMTS13 mutations, V88M and G239V, have been recently reported [34]. A total lack of ADAMTS13 activity in the plasma is thought to be incompatible with life [27], thus, the patients may have very low amounts of ADAMTS13 activity in their plasma, which we were unable to detect with this method.

The patients with acquired TTP presented with a normal ADAMTS13 band, which is consistent with the fact that ADAMTS13 protease genotype and expression are normal, but their activity is lower, due auto-antibodies [11, 14, 41, 45, 52]. The finding that the immunoadsorption of immunoglobulins from the plasma of the patients with acquired TTP also led to the removal of the ADAMTS13 antigen from their samples indicates that the protease is complexed with the auto-antibodies in the circulation of these patients, and that this occurs even during clinical remission.

The majority of the ADAMTS13 assays available today detect ADAMTS13 activity levels in plasma (Table 2). This is performed either by detecting the VWF products resulting from ADAMTS13 cleavage (assays 1–5) or by measuring the residual VWF activity (assays 6–7). The VWF substrate utilized in these assays may be high molecular weight VWF (plasma-derived or recombinant; assays 1–4) or VWF domains or short synthetic peptides (assay 5). Two multicenter studies evaluating methods 1, 3–5, and 6–7 showed that all assays were able to detect severely ADAMTS13-deficient plasma samples and indicated that methods 1, 6, and 7 were the most consistent and reliable methods [48, 49]. A recent smaller study evaluating the FRETS-VWF73 method (assay 5) showed that this is a reliable assay which provides results in good accordance with other methods [26].

Few recent studies have shown that ADAMTS13 antigen was detectable in plasma by ELISA (assay 8 in Table 2). ELISA assays are a valuable complement to the ADAMTS13 activity assays, and offer a fast analysis of the ADAMTS13 antigen levels in plasma samples using antibodies directed to ADAMTS13. These assays are able to distinguish ADAMTS13-deficient TTP plasma from normal plasma samples. Other methods are capable of detecting anti-ADAMTS13 auto-antibodies (Table 2, assays 8–9). Mutational analysis can be carried out by polymerase chain reaction (PCR) in order to detect mutations in the *ADAMTS13* gene (assay 11 in Table 2).

In the present study, we developed a qualitative, semi-quantitative assay capable of detecting ADAMTS13 antigen and anti-ADAMTS13 auto-antibodies in plasma and,

Table 2 ADAMTS13 assays

ASSAY	PRINCIPLE	REFERENCE
Detection of ADAMTS13 activity		
Detection of VWF cleavage products		
1. VWF multimer structure analysis	Detection of the breakdown of high-molecular-weight VWF	[15, 23]
2. Immunoblotting of VWF	Detection of cleavage products of native VWF or recombinant VWF domains	[37, 50]
3. IRMA	Detection of VWF cleavage products	[32]
4. Flow assay	Detection of the breakdown of ULVWF-platelet strings attached to endothelial cells	[1]
5. Various methods using VWF domains or short synthetic VWF peptides as the substrate, such as the FRETS-VWF73 assay	Detection of cleavage products of the VWF domains or VWF peptides	[9, 18, 22, 25, 59–61]
Detection of VWF residual activity		
6. Collagen binding	Detection of VWF binding to collagen; binding correlates to VWF multimer size	[17]
7. Ristocetin cofactor activity	Detection of platelet aggregates; VWF ability to induce platelet aggregates in the presence of ristocetin correlates to multimer size	[7]
Detection of ADAMTS13 antigen and auto-antibodies		
8. ELISA	Detection of ADAMTS13 antigen or anti-ADAMTS13 auto-antibodies	[11, 38, 39, 41, 54]
9. Immunoblotting	Detection of anti-ADAMTS13 auto-antibodies	[57]
10. Present assay (immunoblotting)	Detection of ADAMTS13 antigen and size and (indirectly) of auto-antibodies	–
Mutation analysis		
11. PCR	Detection of mutations in the *ADAMTS13* gene	[27]

IRMA: immunoradiometric assay; FRET: fluorescence resonance energy transfer; FRETS-VWF73: a 73-amino-acid-long synthetic peptide which provides a minimal substrate for ADAMTS13 [24] that has been made fluorogenic; PCR: polymerase chain reaction

thus, distinguishing between TTP and normal plasma, as well as distinguishing between congenital and acquired TTP. All patients with congenital TTP, regardless of their ADAMTS13 mutations, presented with undetectable levels of ADAMTS13 antigen, whereas patients with acquired TTP presented a normal phenotype. Heterozygous carriers of ADAMTS13 mutations also revealed a normal ADAMTS13 antigen band. Although not a quantitative method like the above-mentioned ELISA assays, the present assay offers the advantage of not only demonstrating the presence or absence of ADAMTS13 antigen, but also the molecular size of ADAMTS13 present in plasma samples. Furthermore, this assay was able to show the presence of anti-ADAMTS13 antibodies indirectly by performing immunoadsorption of plasma immunoglobulins prior to immunoblotting. In conclusion, this assay is able to distinguish between normal and TTP plasma, and also between congenital and acquired TTP. It is easy to perform and can be used at any hospital laboratory routinely using SDS-PAGE electrophoresis and immunoblotting, and offers a complement to existing ADAMTS13 methods in the diagnosis of TTP.

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