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# Ticlopidine- and clopidogrel-associated thrombotic thrombocytopenic purpura (TTP): review of clinical, laboratory, epidemiological, and pharmacovigilance findings (1989–2008)

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**Thrombotic thrombocytopenic purpura (TTP) is a fulminant disease characterized by platelet aggregates, thrombocytopenia, renal insufficiency, neurologic changes, and mechanical injury to erythrocytes. Most idiopathic cases of TTP are characterized by a deficiency of ADAMTS13 (a disintegrin and metalloprotease, with thrombospondin-1-like domains) metalloprotease activity. Ironically, use of anti-platelet agents, the thienopyridine derivates clopidogrel and ticlopidine, is associated with drug induced TTP. Data were abstracted from a systematic review of English-language literature for thienopyridine-associated TTP identified in MEDLINE, EMBASE, the public website of the Food and Drug Administration, and abstracts from national scientific conferences from 1991 to April 2008. Ticlopidine and clopidogrel are the two most common drugs associated with TTP in FDA safety databases. Epidemiological studies identify recent initiation of anti-platelet agents as the most common risk factor associated with risks of developing TTP. Laboratory studies indicate that most cases of thienopyridine-associated TTP involve an antibody to ADAMTS13 metalloprotease, present with severe thrombocytopenia, and respond to therapeutic plasma exchange (TPE); a minority of thienopyridine-associated TTP presents with severe renal insufficiency, involves direct endothelial cell damage, and is less responsive to TPE. The evaluation of this potentially fatal drug toxicity can serve as a template for future efforts to comprehensively characterize other severe adverse drug reactions.**

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Thrombotic thrombocytopenic purpura (TTP) is a microvascular occlusive disorder characterized by systemic or intrarenal aggregation of platelets, leading to thrombocytopenia and mechanical injury to erythrocytes.[1] Conditions and factors associated with TTP include organ transplantation, infectious diseases, and drugs.[2] The most common drugs reported to the Food and Drug Administration (FDA) in association with TTP are the thienopyridine-derivative anti-platelet agents, ticlopidine and clopidogrel.[3,4] Before 1999, ticlopidine was widely used for prevention of cerebrovascular, cardiovascular, and peripheral vascular complications and following coronary artery stent procedures.[5] Since 2000, owing to concerns over ticlopidine-associated agranulocytosis, clinicians switched to clopidogrel in these settings.[5–9] Herein, we summarize the clinical, laboratory, and epidemiological information on thienopyridine-associated TTP.

## PHARMACOLOGY

Ticlopidine and clopidogrel, thienopyridine derivatives that inhibit platelet aggregation,[5] differ structurally by a carboxymethyl side group. Animal studies and *in vitro* laboratory studies indicate that ticlopidine, but not clopidogrel, is associated with bone marrow toxicity.[5] As all clopidogrel metabolites contain the carboxymethyl side group, the two drugs have no common metabolites.[10] Ticlopidine and clopidogrel are administered orally, requiring hepatic breakdown to an active metabolite to achieve *in vivo* activity. The major therapeutic target of the thienopyridines is one of the adenosine diphosphate receptor types on human platelets, $P2Y_{12}$. Blockade of this receptor impairs adenosine diphosphate-induced platelet aggregation and decreases the propensity for arterial thrombosis.

## EPIDEMIOLOGY

Epidemiological investigations identified a strong association of TTP with ticlopidine (Table 1). The first cases of ticlopidine-associated TTP were identified in 1991 at an apheresis center in Paris.[11] In 1998, a survey of apheresis centers supplemented by FDA adverse event reports identified 60 cases of ticlopidine-associated TTP; one-third had died from the TTP.[12] Most patients had received between 2–12 weeks of ticlopidine.[12,13] Subsequently, after the introduction of coronary artery stent procedures, additional ticlopidine-associated TTP cases were identified at interventional cardiology laboratories and therapeutic plasma exchange (TPE) centers.[13,14] Two surveys of interventional cardiology laboratories that had placed coronary artery stents in 8000 and 45,000 persons identified rates of TTP after ticlopidine administration of 1 in 1600 and 1 in 5000 patients, respectively.[15,16] These findings placed ticlopidine as the drug with the highest reported rate of TTP.

Recent investigations evaluated the association of clopidogrel with TTP. The first two cases were identified by the directors of apheresis centers in 1998, shortly after the drug received FDA approval.[17] In 2000, eleven cases of TTP after administration of clopidogrel were identified at apheresis centers in six cities.[17] By 2004, 37 cases of

**Table 1 | Comparison of basic science, epidemiological, clinical, and pharmacovigilance findings for ticlopidine- versus clopidogrel-associated TTP**

| Category | Ticlopidine-associated TTP | Clopidogrel-associated TTP |
|---|---|---|
| *Basic science*[20] | | |
| Probable underlying pathophysiology | Antibody to ADAMTS13 and microvascular endothelial cell damage | Microvascular endothelial cell damage |
| High molecular weight vWF identified during the acute TTP phase | Yes | Yes |
| ADAMTS13 deficiency during the acute TTP phase | Yes | No |
| Functional IgG inhibitors to ADAMTS13 identified during acute phase | Yes | No |
| *Clinical*[20] | | |
| Usual time period for onset | Two to 12 weeks after drug initiation | Within 2 weeks of drug initiation |
| Renal insufficiency | Mild to none | Severe |
| Thrombocytopenia | Severe | Mild |
| Survival after plasma exchange | >90%, usually within days of initiation of plasma exchange | 70%, often takes several weeks of plasma exchange |
| Survival without plasma exchange | 30% | 70% |
| Spontaneous relapse | Occasional | Infrequent |
| Likelihood of relapse occurring with exposure to the other thienopyridine | High | Low |
| *Epidemiological* | | |
| Epidemiological studies identifying cases of TTP after thienopyridine administration | Surveys of directors of interventional cardiology laboratories as well as directors of therapeutic plasma exchange centers ($n$=33 cases)[26,29] | Surveys of directors of therapeutic plasma exchange centers ($n$=13 cases)[5,31] |
| Estimated incidence based on information included in the FDA-approved package insert | 0.01–0.02%[37] | 0.0001%[18] (threefold greater than estimated incidence of idiopathic TTP) |
| Population-based case–control studies | None | Recent initiation of anti-platelet agents (clopidogrel, aspirin, or dipyridamole) is associated with 19.8-fold increased risk of developing TTP ($n$=86 cases; 177 age- and gender-matched controls)[41] |
| *Pharmacovigilance* | | |
| Number of thienopyridine-associated TTP cases identified in the first year of marketing of the relevant drug | 4 (year=1991)[11] | 2 (year=1999)[38,39] |
| Number of cases included in the largest case series | 98 patients[14] | 50 patients[3] |
| Year of FDA approval | 1991 (current sales are $100,000)[9] | 1998 (current sales are $7.3 billion)[9] |
| Time from FDA approval to identification of first cases | 0 years (4 cases) (1991) | 1 year (2 cases) (1999) |
| Time from FDA approval to reporting of first case series | 7 years (1998) | 1.5 years (2000) |
| Rank in FDA MedWatch database in association with drug-associated TTP reports (1998–2006) | First—overall (first in the years 1998 and 1999) | Second—overall (first since 2000) |
| Advisories from the FDA | Package insert warning (1995)[40]<br>Black box warning (1998)[37] | Package insert warning (2000)[18] |
| 'Dear Doctor' warnings describing drug-associated TTP mailed by the pharmaceutical supplier | 1998 | 2000 |

ADAMTS13, a disintegrin and metalloprotease, with thrombospondin-1-like domains; FDA, Food and Drug Administration; TTP, thrombotic thrombocytopenic purpura.

clopidogrel-associated TTP had been reported to the FDA.[3] The pharmaceutical supplier reported an estimated incidence rate of 12 TTP cases per million clopidogrel-treated patients,[18] three times the background rate for TTP in the general population.[19]

## CLINICAL FEATURES

Thienopyridine-associated TTP is characterized by two clinical syndromes (Figure 1a). Most cases of ticlopidine-associated TTP and a minority of clopidogrel-associated TTP cases present with severe thrombocytopenia, microangiopathic hemolytic anemia, markedly elevated serum levels of lactate dehydrogenase, and normal renal function; and they occur between 2 and 12 weeks after initiation of thienopyridine therapy. Most cases of clopidogrel-associated TTP and a minority of ticlopidine-associated TTP cases present with mild thrombocytopenia, microangiopathic hemolytic anemia, mildly elevated serum levels of lactate dehydrogenase, and marked renal insufficiency. Onset usually occurs within 2 weeks of thienopyridine initiation.[3,17,20] Both syndromes differ from those reported for other drug-associated TTP syndromes.[2] Thrombotic microangiopathy associated with the calcineurin inhibitors gemcitabine and mitomycin-C is dose dependent, occurs after several weeks or months of use, and is attributed to the cumulative toxic effects on vascular endothelium.[2] Renal dysfunction is generally present, with many patients requiring hemodialysis. TPE is not effective.[2] Thrombotic microangiopathy developing after organ transplantation is associated with calcineurin inhibitors. The most common treatment strategy is discontinuation of the drug.[2] Although quinine-associated TTP/HUS is antibody mediated, the antibodies are directed against granulocytes, lymphocytes, endothelial cells, or platelet glycoprotein Ib/IX or IIb/IIIa complexes.[21] The syndrome can occur after ingestion of a single tablet of quinine in previously exposed persons, and is characterized by neurologic complications, thrombocytopenia, and hemolysis. Renal failure is absent occasionally. Treatment includes discontinuation of quinine, TPE, and hemodialysis.

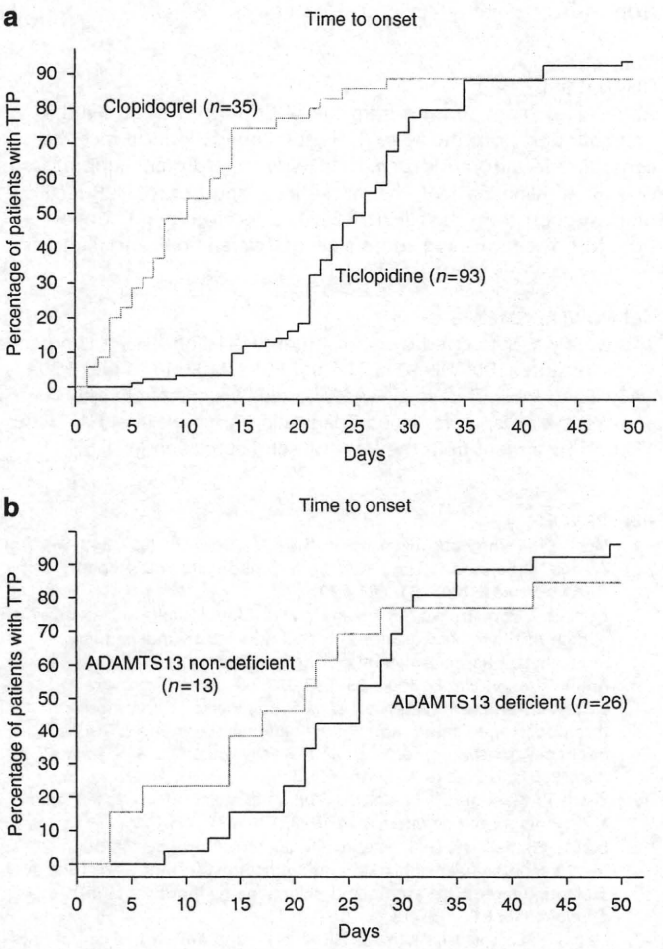


**Figure 1 | Duration of thienopyridine exposure prior to TTP onset.** (**a**) Thienopyridine-associated thrombotic thrombocytopenic purpura (TTP) onset: ticlopidine versus clopidogrel ($P = 0.0016$). (**b**) Thienopyridine-associated TTP onset: ADAMTS13 (a disintegrin and metalloprotease, with thrombospondin-1-like domains) deficient (<15%) versus near-normal levels (>15%) of ADAMTS13 activity ($P > 0.05$). This figure has previously been published in Bennett *et al.*[20]

## PATHOPHYSIOLOGY

For most patients with ticlopidine-associated TTP and a minority of patients with clopidogrel-associated TTP, *in vitro* assessments of plasma ADAMTS13 activity show severely diminished activity at the time of TTP onset.[20] Onset of TTP occurs between 2 and 12 weeks after thienopyridine initiation (Figure 1b). Reduced *in vitro* ADAMTS13 activity correlates with deficient ADAMTS13 activity near the surface of stimulated endothelial cells that secrete ULVWF multimers. Plasma from six of seven patients with ticlopidine-associated TTP and from two of eleven patients with clopidogrel-associated TTP contained inhibitors to the ADAMTS13 metalloprotease.[17,22] Failure to process ULVWF multimers seems to lead to the binding of ULVWF to platelets, systemic platelet aggregation, and TTP.[23] After TPE and thienopyridine discontinuation, most patients with ADAMTS13 deficiency, anti-ADAMTS13 autoantibodies, and thienopyridine-associated TTP recover. Plasma exchange may lead to removal of ULVWF multimers, removal of autoantibodies to ADAMTS13, and replacement of the ADAMTS13 with that present in fresh frozen plasma. Clinical findings also require stimulation of endothelial cells to secrete ULVWF. Such a double-insult model is exemplified by the ADAMTS13 knockout mouse, which requires endothelial cell stimulation to evoke a TTP-like microvascular thrombosis.[24] In genetically predisposed individuals, thienopyridine may stimulate an autoimmune anti-ADAMTS13 antibody response and microvascular endothelial injury. Ticlopidine and clopidogrel are protein-bound in plasma and can function as haptens capable of eliciting IgE and IgG antibody formation.[25] However, they do not directly bind to ADAMTS13 and stimulate production of antibodies that inhibit ADAMTS13

enzyme activity. Anti-ADAMTS13 antibodies generated in a fraction of thienopyridine-treated patients do not require the presence of the drug (or metabolite).[17,22,26] Thienopyridine/anti-ADAMTS13 antibodies are analogous to warm autoantibodies against red blood cell antigens that emerge in a subset of patients treated with the antihypertensive agent, α-methyldopa.[27] Binding of thienopyridines to $P2Y_{12}$ molecules on different cell types may, in a fraction of exposed individuals, initiate anomalous intracellular signaling patterns or provoke antibody production against the haptenic thienopyridine–$P2Y_{12}$ protein complex on cell surfaces. Malfunction or injury to lymphocytes, CD34+ stem cells, or endothelial cells may result.

For most clopidogrel-associated and a minority of ticlopidine-associated TTP patients, the syndrome is characterized by mild thrombocytopenia, microangiopathic hemolytic anemia, and marked renal insufficiency.[20,28] Onset of TTP is generally within 2 weeks of thienopyridine initiation (Figure 1a). Most cases have ULVWF in their plasma and near-normal levels of plasma ADAMTS13 metalloprotease activity during the acute phase of the syndrome, suggesting endothelial cell injury or stimulation with release of ULVWF.[20] Thienopyridines may bind to $P2Y_{12}$ receptors (with or without anti-thienopyridine antibodies) on CD34+ stem cells, altering cell proliferation and differentiation. Interaction of thienopyridines with endothelial cells has been shown to result in nitric oxide and possibly prostacyclin ($PGI_2$) generation.[29,30] At least some thienopyridine binding to human endothelial cells is likely to be through the $P2Y_{12}$ receptors on these cells. In a few thienopyridine-treated patients, the endothelial cell response to the thienopyridine (with or without antibody) attachment may be a combination of cell injury, excessive secretion of ULVWF multimeric strings, or apoptosis.[23]

## THERAPY

All patients who develop thienopyridine-associated TTP should have prompt plasma exchange.[1,3,12,17,31,32] Plasma exchange is continued until the goals of resolution of neurologic symptoms, improvement of LDH to near normal, and achieving and maintaining for 2–3 days a platelet count of 150,000/$mm^3$ are achieved.[33] After this, plasma exchange may be either discontinued or reduced in frequency.[32] Among thienopyridine-associated TTP patients who have antibody-mediated ADAMTS13 deficiency, a few days of plasma exchange are required. Although patients generally recover without permanent organ damage, a spontaneous relapse occurs occasionally. Among thienopyridine-associated TTP patients without autoantibodies against ADAMTS13, several weeks of plasma exchange are often required and spontaneous relapses are rare.[3,14,17,20] One report describes a patient with a drug-eluting coronary artery stent who developed TTP within days of clopidogrel initiation. After the TTP resolved with TPE and clopidogrel discontinuation, the patient was re-challenged with ticlopidine and did not experience a TTP relapse.[34]

## CONCLUSIONS

Thienopyridine-associated TTP has served as the focus of intensive scientific investigation over the past two decades.[12,14,15,17,20,22,23,26,35,36] As with idiopathic TTP, most thienopyridine-associated TTP cases are associated with autoantibodies that inhibit the plasma metalloprotease, ADAMTS13. For these individuals, TTP onset usually occurs within 2–12 weeks after initiation of ticlopidine (rarely clopidogrel) and resolves rapidly with TPE. For a minority of cases of thienopyridine-associated TTP, autoantibodies directed against ADAMTS13 metalloprotease have not been implicated. For these persons, the syndrome occurs within 2 weeks of initiating clopidogrel (rarely ticlopidine) and is less responsive to TPE. Prominent warnings in FDA-approved labels for ticlopidine and clopidogrel describe clinical findings, and the importance of timely initiation of plasma exchange for patient who develop this toxicity. The clinical, laboratory, and epidemiological evaluation and related pharmaceutical safety interventions for thienopyridine-associated TTP can serve as a template for future efforts to investigate and protect patients against harm from other severe adverse drug reactions.

## DISCLOSURE

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bjh research paper

# Pregnancy-induced thrombocytopenia and TTP, and the risk of fetal death, in Upshaw–Schulman syndrome: a series of 15 pregnancies in 9 genotyped patients

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## Summary

Upshaw–Schulman syndrome (USS) is a congenital thrombotic thrombocytopenic purpura (TTP) due to mutations in the gene that encodes for ADAMTS13 (*ADAMTS13*), but its clinical signs may be mild or absent during childhood. We have identified 37 patients with USS (24 females, 13 males) belonging to 32 families. The nine women from six families who were diagnosed during their first pregnancy are the focus of this report. Six of the nine women had episodes of thrombocytopenia during childhood misdiagnosed as idiopathic thrombocytopenic purpura. Thrombocytopenia occurred during the second–third trimesters in each of their 15 pregnancies, with 16 babies (one twin pregnancy), often followed by TTP. Of 15 pregnancies, eight babies were stillborn or died soon after birth, and the remaining seven were all premature except one, who was born naturally following plasma infusions to the mother that had started at 8 weeks' gestation. All nine USS women had severely deficient ADAMTS13 activity. *ADAMTS13* analyses demonstrated that eight women were compound heterozygotes of Y304C/G525D (2 siblings), R125VfsX6/Q1302X (2 siblings), R193W/R349C (2 siblings), I178T/Q929X, and R193W/A606P; one woman was homozygous for R193W. Only the R193W mutation has been previously reported. These observations emphasize the importance of measuring ADAMTS13 activity in the evaluation of thrombocytopenia during childhood and pregnancy.

**Keywords:** Upshaw–Schulman syndrome, pregnancy, *ADAMTS13* mutation, thrombocytopenia, haemolytic anaemia.

Upshaw–Schulman syndrome [USS, also described as congenital thrombotic thrombocytopenic purpura (TTP)] is a congenital deficiency of ADAMTS13 (A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motifs-13) activity due to *ADAMTS13* gene mutations (Kinoshita *et al*, 2001; Levy *et al*, 2001; Kokame *et al*, 2002). When ADAMTS13 activity is deficient, unusually large von Willebrand factor (VWF) multimers accumulate in the circulation that can cause platelet thrombi under high shear stress of the microcirculation (Yagi *et al*, 2001; Moake, 2002). In 2005, the presence of roughly 100 patients with USS in 80 families worldwide was estimated (Kremer-Hovinga *et al*, 2005). But still USS patients may often be overlooked in clinical practice. Therefore, the frequency of USS is presumably underestimated.

Acquired TTP, caused by neutralizing or non-neutralizing autoantibodies to ADAMTS13 that reduce plasma ADAMTS13 activity, is a disorder with an incidence of 1·7 per million population; 73% of the patients were women and most women were of child-bearing age (Terrell *et al*, 2005). Although acquired TTP and USS have some similarities, USS has important differences in clinical features from acquired TTP (Furlan & Lämmle, 2001; Fujimura *et al*, 2002).

During the past 10 years, we have diagnosed 37 patients with USS by assaying ADAMTS13 activity and its inhibitor titres in the laboratory of Nara Medical University. Further, analysis of the natural history and *ADAMTS13* mutations in these patients showed that severe neonatal jaundice requiring exchange blood transfusions, a classic hallmark of USS, was seen in only 16 (43%) of 37 patients. Twenty-nine (79%) of the 37 patients had a history of thrombocytopenia during childhood that was misdiagnosed as idiopathic thrombocytopenic purpura (ITP). Nine women from six families were first diagnosed during pregnancy. Further, we documented that thrombocytopenia inevitably developed during the second or third trimester of all 15 pregnancies in these nine women. Often, the initial isolated thrombocytopenia was followed by overt signs of microangiopathic haemolytic anaemia and TTP. Notably, of 15 pregnancies (one twin pregnancy), eight babies were stillborn or died soon after birth, and seven babies were all premature but survived. Only one mature baby was born at full term following plasma infusions to the mother that had started at 8 weeks gestation. Six of these nine USS-women had episodes of severe to mild thrombocytopenia during childhood that had been incorrectly diagnosed as ITP.

Because of the important association between USS and pregnancy we report the detailed natural history and clinical characterization of these nine women as well as their *ADAMTS13* mutations. Our experience emphasizes the importance of measuring ADAMTS13 activity as part of the evaluation of thrombocytopenia during childhood or pregnancy.

## Patients, materials and methods

### *Patients with USS*

Between July 1998 and April 2008, 37 patients with USS (24 females and 13 males) belonging to 32 different families were identified at Nara Medical University. Severe neonatal jaundice that required exchange blood transfusion, a hallmark of USS, was found in 16 (43%) of these 37 patients. Notably, nine USS patients belonging to 6 families (designated as K, L, M, O, R and Z) had been pregnant and they all developed thrombocytopenia, often followed by TTP, at the second or third trimester. Thus, these 9 patients were extensively studied here. None of these nine women had received contraceptive agents before their pregnancies. It is also important to note that the remaining 15 USS women had no pregnancies. These data will be published elsewhere.

### *Assays of ADAMTS13 activity and ADAMTS13 inhibitor*

Plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor were measured by both a classic VWF multimer assay (Furlan *et al*, 1998) and a chromogenic ADAMTS13-activity-enzyme-linked immunosorbent assay (ADAMTS13 act-ELISA) (Kainos Inc., Tokyo) (Kato *et al*, 2006). The ADAMTS13 activity of pooled normal plasma was defined as 100%, and the detection limits of classic VWF multimer assay and the act-ELISA were 3% and 0·5% of the normal controls respectively. The ADAMTS13 inhibitor titres were expressed in Bethesda units (BU), where one inhibitor unit was defined as the amount necessary to reduce ADAMTS13 activity to 50% of the control level. A titre of ≥0·5 BU/ml in both the assays was considered to be significant.

### *Assays of ADAMTS13 antigen*

Plasma levels of ADAMTS13 antigen were determined by a quantitative sandwich antigen ELISA (ag-ELISA) by using two anti-ADAMTS13 monoclonal antibodies (mAbs); a neutralizing mAb A10 as the coating antibody and a non-neutralizing mAb C7 labeled with horseradish peroxidase (HRP) as the detection antibody (Yagi *et al*, 2007). The epitopes recognized by mAbs A10 and C7 were determined to reside in the disintegrin-like domain and the 7th and 8th thrombospondin type-1 domains respectively (Uemura *et al*, 2005). The ADAMTS13 antigen of pooled normal plasma was defined as 100%, and the detection limit was 0·1% of the normal controls.

Further, ADAMTS13 antigen was analysed by Western blot under reducing conditions, quantitatively and qualitatively (Ishizashi *et al*, 2007). Briefly, this was performed as the follows. An aliquot (2 μl) of diluted or undiluted plasma sample per lane were separated by sodium dodecyl sulfate-5% polyacrylamide gel electrophoresis (SDS-5%PAGE) under reducing conditions. After electrophoresis, the proteins were electrophoretically blotted onto polyvinylidene difluoride

(PVDF) microporous membranes. We probed the blots for ADAMTS13 antigen with WH2-11-1 as the primary mAb, followed by secondary staining with HRP-conjugated goat anti-mouse IgG (Kirkegaard & Perry Lab, Gaithersburg, MO, USA). The epitope of mAb WH2-11-1 resides in the fourth thrombospondin type-1 domain (Soejima *et al*, 2006). After incubation with Western Lighting Chemiluminescence Reagent (PerkinElmer Life Sciences, Shelton, CT), the blots were exposed to X-ray film. Densitomeric analysis of ADAMTS13 antigen was performed for the 190-kD band using the National Institutes of Health (NIH) image J (developed by NIH, http://rsb.Info.gov/noh-image/). The detection limit of plasma ADAMTS13 antigen by this method was 3% of the normal controls.

### *ADAMTS13 gene analysis*

All DNA analyses were performed with the permission of Ethics Committees of both the sample-collecting hospitals and the institute that performed the gene analysis. Written informed consent was obtained from all patients. The nucleotide sequences of the entire 29 exons of *ADAMTS13*, including the intron–exon boundaries, were determined by direct sequencing of polymerase chain reaction (PCR) products, as previously described in detail (Kokame *et al*, 2002; Matsumoto *et al*, 2004). All the disease-causing *ADAMTS13* mutations reported here were excluded as common polymorphisms by screening 96 individuals in the Japanese general population.

## Results

The natural history, clinical characterization, and ADAMTS13 activity of the 9 women with USS and their family members are summarized in Table I. Detailed descriptions of these patients are provided in the text.

### *Family K, clinical data*

Proposita K-3, born in 1976, was the older of 2 siblings born to non-consanguineous parents (Fig 1, top). She had no history of severe neonatal jaundice. At the age of 6 years, she developed nasal bleeding with thrombocytopenia and was diagnosed with ITP. She then had repeated episodes of thrombocytopenia during childhood. Her first pregnancy was at the age of 27 years. The initial platelet count was normal ($180 \times 10^9$/l), but at 24 weeks gestation the platelet count decreased to $17 \times 10^9$/l. She received steroid therapy and then high-dose immunoglobulin G (IVIg) infusions in a local hospital without clinical improvement. She subsequently developed microangiopathic haemolytic anaemia with neurological and renal abnormalities, was diagnosed with TTP, and treated with plasma exchange. Her fetus died at 25 weeks gestation and was removed by Caesarean section; necrotic lesions and intravascular thrombi were identified in a large area of the placenta and also the uterus, necessitating hysterectomy. Following hysterectomy and plasma exchange (twice during 2 d), a normal platelet count ($\geq 150 \times 10^9$/l) was achieved, but a few weeks after cessation of plasma exchange the platelet count dropped again requiring an additional plasma exchange treatment. Following recovery, her plasma ADAMTS13 activity and ADAMTS13 inhibitor were assayed by the VWF multimer method, demonstrating a severe deficiency of ADAMTS13 activity but without an inhibitor, suggesting a diagnosis of USS.

At the same time, her younger sister (K-4), born in 1978 (age 25 years), was a primigravida at 22 weeks gestation, and she also had mild thrombocytopenia ($59 \times 10^9$/l) and anaemia (Hb 91 g/l). She had a history of severe neonatal jaundice that required exchange blood transfusion. At the age of 4 years, she had an episode of severe thrombocytopenia with bleeding (site unknown) that required blood transfusion, and was diagnosed as ITP. Because of her elder sister's diagnosis of USS, this patient was tested for ADAMTS13 activity and ADAMTS13 inhibitor, demonstrating the same results as her elder sister. Thus, prophylactic FFP infusions (320 ml every 2 weeks) were instituted to maintain pregnancy. Under these circumstances, at 29 weeks gestation the patient had a premature boy by Caesarean section.

After the diagnosis of USS, both the siblings had receiving 320 ml of FFP infusions every 3 weeks. In 2005, the plasma levels of ADAMTS13 activity in both K-3 and K-4 were examined by the act-ELISA, and indeed they were shown to be severely deficient (<0·5% of the normal) but without its inhibitors (<0·5 BU/ml).

### *ADAMTS13 analysis (heterozygous Y304C/G525D mutation)*

The *ADAMTS13* mutations of both the affected siblings (K-3 and K-4) were shown to be the compound heterozygotes of Y304C (*c.* 911A > G, exon 8) and G525D (*c.* 1574G > A, exon 13), and their parents were heterozygous carriers of either of the mutations. Five common single nucleotide polymorphisms (SNPs) found in the two affected siblings are listed in Table II.

Plasma levels of ADAMTS13 antigen by ag-ELISA were 0·4% of normal in both the affected siblings, and 31% and 36% in their father and mother respectively. Further, plasma levels of the respective ADAMTS13 antigen by Western blot were shown to be 5·1%, 4·8%, 39% and 34%. Thus, the Y304C/G525D mutation was apparently secreted into the plasma, but with a markedly reduced level (Fig 1, top right and Fig 2).

### *Family L, clinical data*

Proposita L-2, born in 1967, was the second of five siblings to non-consanguineous parents. Of her five siblings, the first child died at 20 weeks gestation with a diagnosis of intrauterine fetal death of unknown aetiology, and two brothers (the third and fourth siblings) were apparently healthy. She had no



Table I. Clinical characterization of 9 female patients with Upshaw–Schulman Syndrome who developed TTP-bouts during pregnancy.

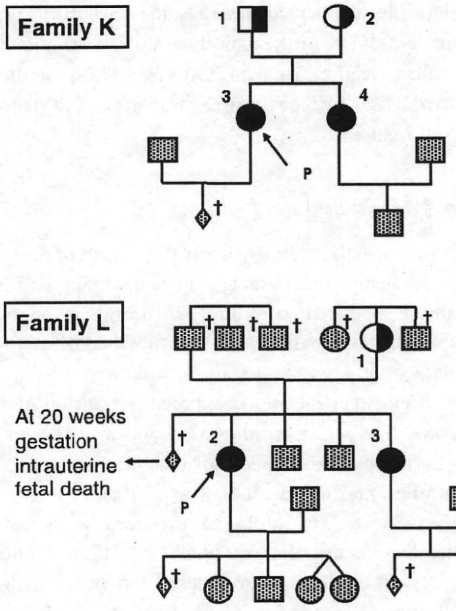
| | | | Newborn period | Childhood | Adulthood (pregnancy) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Before | After | | | | | Therapy and outcome | | |
| Number | USS-patients | Year of birth | Exchange blood transfusion for severe jaundice | Clinical diagnosis (age) | Clinical diagnosis | Age (years) | Gestation (weeks) | Therapy | Gestation (weeks) | Diagnosis | Babies | Mothers | Year of diagnosis for USS by ADAMTS13 assay |
| 1 | K-3 | 1976 | No | ITP (6 years) | ITP | 27 | 24 | ST + IVIg | 25 | TTP | IUFD at 25 weeks gestation | PE (2 times), CS, necrosis of placenta and uterus, hysterectomy, remission | 2003 |
| 2 | K-4 | 1978 | Yes | ITP (4 years) | ITP | 25 | 20 | FFP infusions | 29 | ITP | Live-birth at 29 weeks gestation, premature baby | CS under FFP infusions, remission | 2003 |
| 3 | L-2 | 1967 | No | None | None | 27 | 27 | Conservative | 28 | HELLP syndrome or APS | IUFD at 27 weeks gestation | CS, remission | – |
| | | | | | APS | 28 | 3rd trim. | Aspirin | 37 | APS | Live-birth at 37 weeks gestation under aspirin intake, Premature baby | CS, remission | – |
| | | | | | APS | 30 | 3rd trim. | Aspirin | 32 | APS | Live-birth at 37 weeks gestation under aspirin intake, Premature baby | CS, remission | – |
| | | | | | APS | 33 | 3rd trim. | Aspirin | 32 | APS | Live-birth at 32 weeks gestation under aspirin intake, Premature twins | CS, remission | 2003 |
| 4 | L-3 | 1972 | No | ITP (3 years) | ITP | 25 | 24 | ST | 25 | Evans syndrome | Stillbirth at 25 weeks gestation | ST, remission after vaginal delivery | – |
| | | | | | ITP | 27 | 23 | PE, Haemodialysis | 24 | HUS | Live-birth of premature baby at 24 weeks gestation, but it died soon after delivery. | PE (2 times) and haemodialysis, Remission after vaginal delivery | 2003 |
| 5 | M-3 | 1969 | No | None | None | 33 | 17 | PE | 20 | TTP | Miscarriage at 20 weeks gestation | PE (2 times), remission | 2003 |

Table I. (Continued).

| | | | Newborn period | Childhood | Adulthood (pregnancy) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Before | After | | | | Therapy and outcome | | |
| Number | USS-patients | Year of birth | Exchange blood transfusion for severe jaundice | Clinical diagnosis (age) | Clinical diagnosis | Age (years) | Gestation (weeks) | Therapy | Gestation (weeks) | Diagnosis | Babies | Mothers | Year of diagnosis for USS by ADAMTS13 assay |
| 6 | M-4 | 1971 | No | None | None | 30 | 28 | CS | | HELLP syndrome | Stillbirth at 28 weeks gestation | CS, remission | – |
| | | | | | | 31 | 33 | Conservative | 36 | ITP | Live-birth at 36 weeks gestation | CS, remission | 2003 |
| 7 | O-4 | 1958 | No | ITP (5 years) | ITP | 26 | 23 | Conservative | 25 | ITP | Live-birth of premature baby at 25 weeks gestation, but it died soon after birth | TTP-bout after vaginal delivery, PE (2 times), remission | – |
| | | | | | Chronic relapsing TTP | 31 | 8 | FFP infusions | 39 | Chronic relapsing TTP | Live-birth weighed 2·984 kg at 39 weeks gestation | Natural delivery | 2004 |
| 8 | R-5 | 1982 | No | ITP (8 months) | ITP | 23 | 23 | Conservative | 30 | TTP (after delivery) | IUFD at 30 weeks gestation | TTP-bout after CS, PE (8 times) and ST, remission | 2005 |
| 9 | Z-3 | 1971 | No | ITP (7 years) | ITP | 25 | 12 | Conservative | 32 | TTP (after CS) | Live-birth at 32 weeks gestation, Premature baby | TTP-bout after CS, PE (times unclear), remission | 1998 |

USS, Upshaw–Schulman Syndrome; ITP, idiopathic thrombocytopenic purpura; TTP, thrombotic thrombocytopenic purpura; HUS, hemolytic uremic syndrome; APS, anti-phospholipid syndrome; ST, steroid; FFP, fresh frozen plasma; IVIg, high dose immunoglobulin G; HELLP, haemolysis, elevated liver enzyme and low platelet count; CS, Caesarean section; IUFD, interuterine fetal death.




| | ADAMTS13 activity (%) | | ADAMTS13 antigen (%) | *ADAMTS13* gene | | |
|---|---|---|---|---|---|---|
| | act-ELISA | VWFM | ag-ELISA | Tyr304 | Gly525 | Haplotype |
| K-1 | 22 | 31 | 62 | Y/C | G/G | YG/CG |
| K-2 | 26 | 36 | 58 | Y/Y | G/D | YG/YD |
| K-3 | <0·5 | <3 | 0·4 | Y/C | G/D | CG/YD |
| K-4 | <0·5 | <3 | 0·4 | Y/C | G/D | CG/YD |

| | ADAMTS13 activity (%) | | ADAMTS13 antigen (%) | *ADAMTS13* gene | | |
|---|---|---|---|---|---|---|
| | act-ELISA | VWFM | ag-ELISA | Arg125 | Gln1302 | Haplotype |
| L-1 | 48 | 76 | 58 | R/fs | Q/Q | RQ/fsQ |
| L-2 | <0·5 | <3 | <0·1 | R/fs | Q/X | fsQ/RX |
| L-3 | <0·5 | <3 | <0·1 | R/fs | Q/X | fsQ/RX |

Fig 1. Pedigree and ADAMTS13 analyses of USS-Family K and L. Squares and circles indicate males and females, respectively, and arrows with P indicate the proposita. Filled symbols represent USS-patients. The half-filled symbols represent asymptomatic carriers. The cross indicates deceased. The ADAMTS13 activity by both act-ELISA and VWF multimers are shown as a percentage of the normal control. The ADAMTS13 antigen by ag-ELISA is shown as a percentage of the normal control. Mutations found in *ADAMTS13* are shown as one-letter amino acid abbreviations.

history of severe neonatal jaundice or childhood thrombocytopenia. She was a primigravida at the age of 27 years, and her platelet count was $180 \times 10^9$/l at 6 weeks gestation, which dropped to $38 \times 10^9$/l at 27 weeks, complicated with acute renal failure, followed by intrauterine fetal death. She was suspected to have HELLP (Hemolysis, Elevated Liver enzyme, and Low Platelet count) syndrome or anti-phospholipid antibody syndrome. Interestingly, soon after Caesarean section her platelet count returned to normal. Because of this episode, her physician recommended that she took a low-dose aspirin (81 mg/d) whenever she had thrombocytopenia in association with pregnancy. At the age of 28 years, she had her second pregnancy, and again developed thrombocytopenia ($21 \times 10^9$/l) and proteinuria during the third trimester, and low-dose aspirin was administered. She delivered by Caesarean section at 37 weeks gestation. After delivery, her platelet count soon returned to normal ($150 \times 10^9$/l). At the age of 30 years, she had her third pregnancy and was treated similarly as above, and was delivered by Caesarean section at 37 weeks gestation. Finally, at the age of 33 years, she had her fourth pregnancy with twins. Even with the administration of low-dose aspirin, she developed haemolytic anaemia with thrombocytopenia ($53 \times 10^9$/l) at 30 weeks gestation, which was suspected to be Coombs'-negative Evans' syndrome and therefore she was treated with oral steroids and IVIg therapy. Her platelet count did not increase, she developed generalized oedema and pleural effusions at 32 weeks gestation, and her premature twins were delivered by Caesarean section. One week after delivery, her platelet count spontaneously increased to $200 \times 10^9$/l. Since then, she has never taken steroid or aspirin.

The younger sister (L-3), born in 1972, had no history of severe neonatal jaundice. At the age of 3 years, she had mild thrombocytopenia ($70 \times 10^9$/l) at the time of a respiratory infection, and was diagnosed with ITP. At the age of 25 years, she was a primigravida, and at 24 weeks gestation developed proteinuria and thrombocytopenia ($20 \times 10^9$/l) that was suspected to be Evans' syndrome and was treated with steroids. Then she had a stillbirth at 25 weeks gestation. After delivery, her platelet count soon recovered to normal. She had her second pregnancy when aged 27 years, and at 23 weeks gestation she developed thrombocytopenia ($14 \times 10^9$/l) and gross hematuria accompanied by renal failure. She was suspected to have haemolytic-uremic syndrome (HUS) and was treated with plasma exchange for 2 d (once per day) and haemodialysis. Despite this intensive care, she showed no clinical improvement, and at 24 weeks gestation she delivered a premature baby transvaginally but it died soon after birth. Her platelet count recovered within 1 week after delivery. These two sisters were referred to Nara Medical University in 2003, and plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor were assayed by the VWF multimer method, demonstrating a severe deficiency of ADAMTS13 activity but without an inhibitor, consistent with a diagnosis of USS. In 2005, the act-ELISA revealed that the plasma levels of ADAMTS13 activity in both L-2 and L-3 were severely deficient (<0·5% of the normal control), but without an inhibitors (<0·5 BU/ml).

**Table II.** Disease-causing mutations and common SNPs of *ADAMTS13 gene* in 9 patients with Upshaw–Schulman syndrome.

| Patients with USS | Exon/ Intron | Nucleotides | Amino acid | Remarks |
|---|---|---|---|---|
| K-3 | exon 8 | 911 A > G | Y304C | disease causing |
| K-4 | | | | mutation |
| | exon 9 | 1016 C > G | T339R | common SNP |
| | exon 12 | 1342 C > G | Q448E | common SNP |
| | exon 13 | 1574 G > A | G525D | disease causing mutation |
| | exon 15 | 1716 G > A | silent | common SNP |
| | exon 16 | 1852 C > G | P618A | common SNP |
| | exon 19 | 2280 C > T | silent | common SNP |
| L-2 | exon 4 | 3712 ins GT | R125VfsX6 | disease causing |
| L-3 | | | | mutation |
| | exon 9 | 1016 C > G | T339R | common SNP |
| | exon 12 | 1342 C > G | Q448E | common SNP |
| | exon 15 | 1716 G > A | silent | common SNP |
| | exon 16 | 1852 C > G | P618A | common SNP |
| | exon 19 | 2280 C > T | silent | common SNP |
| | exon 28 | 3904 C > T | Q1302X | disease causing mutation |
| M-3 | exon 6 | 577 C > T | R193W | disease causing |
| M-4 | | | | mutation |
| | exon 9 | 1045 C > T | R349C | disease causing mutation |
| | exon 19 | 2280 C > T | silent | common SNP |
| O-4 | exon 5 | 533T > C | I178T | disease causing mutation |
| | exon 19 | 2280 C > T | silent | common SNP |
| | exon 22 | 2785 C > T | Q929X | disease causing mutation |
| R-5 | exon 6 | 577 C > T | R193W | disease causing mutation |
| | exon 9 | 1016 C > G | T339R | common SNP |
| | exon 12 | 1342 C > G | Q448E | common SNP |
| | exon 15 | 1716 G > A | silent | common SNP |
| | exon 16 | 1816 G > C | A606P | disease causing mutation |
| | exon 16 | 1852 C > G | P618A | common SNP |
| | exon 19 | 2280 C > T | silent | common SNP |
| Z-3 | exon 6 | 577 C > T | R193W | disease causing mutation |
| | exon 19 | 2280 C > T | silent | common SNP |

### *ADAMTS13 analysis (heterozygous R125VfsX6/Q1302X mutation)*

The *ADAMTS13* mutations were the compound heterozygotes of R125VfsX6 (c.372insGT, exon 4) and Q1302X (*c.* 3904C > T, exon 28) in both the affected siblings (L-2 and L-3). Their parents were heterozygous carriers of either of the mutations. Seven common SNPs found in the two affected siblings are listed in Table II.

Plasma levels of ADAMTS13 antigen by the ag-ELISA were severely deficient in both patients, <0·1% of the normal, and 58% in their mother. The ADAMTS13 antigen analysis by Western blot confirmed these results: <3% of normal in both the patients, 79% in their mother, and no other bands with a molecular weight less than 190 kDa. Thus, neither mutant protein, R125VfsX6 or Q1302X, was present in plasma (Fig 1, bottom right and Fig 2).

### *Family M, clinical data*

Proposita M-3, born in 1969, was the second of four siblings to non-consanguineous parents (Fig 3, top). She had no history of severe neonatal jaundice or thrombocytopenia during childhood. The 1st child in this family died of utero-placental thrombosis at 32 weeks gestation, and the 4th child (M-5) has never been pregnant and is apparently healthy. At the age of 33 years, M-3 was a primigravida and at 17 weeks gestation she developed petechiae and oedema in both the lower extremities, and was diagnosed as TTP at 19 weeks gestation. She was treated with plasma exchange twice for 2 d but eventually miscarried at 20 weeks gestation. Thereafter, her platelet count quickly recovered, but 1 month later her platelet count again dropped to $24 \times 10^9$/l. She received plasma infusion, which increased her platelet count to normal.

Her younger sister (M-4), born in 1971, also had no history of severe neonatal jaundice or thrombocytopenia during childhood. At the age of 30 years, she had her first pregnancy and at 28 weeks gestation she developed thrombocytopenia, hypertension, and proteinuria, suggesting a diagnosis of HELLP syndrome. A Caesarean section was performed, but the child was stillborn. The next year, she had her second pregnancy, and at 33 weeks gestation she developed thrombocytopenia, and Caesarean section was performed at 36 weeks gestation, delivering a live female infant.

Both the affected siblings (M-3 and M-4) in this family were diagnosed with USS in 2003 after determination of ADAMTS13 activity and ADAMTS13 inhibitor by VWF multimer assay. In 2005, the act-ELISA revealed that the plasma levels of ADAMTS13 activity in both M-3 and M-4 were indeed severely deficient (<0·5% of the normal), without inhibitors (<0·5 BU/ml).

### *ADAMTS13 analysis (heterozygous R193W/R349C mutation)*

The *ADAMTS13* mutations were compound heterozygotes of R193W (*c.* 577C > T, exon 6) and R349C (*c.* 1045C > T, exon 9) in both affected siblings (M-3 and M-4); their parents were heterozygous carriers of either of the mutations. One common SNP was found in these two affected siblings (Table II).

Plasma levels of ADAMTS13 antigen by the ag-ELISA were 8·6% and 7·7% of the normal in the patients, 57%, and 63% in their parents. Further, plasma levels of ADAMTS13 antigen by Western blot were 24% and 23% in two patients, and 45% and 43% in their parents. Thus, the R193W/R349C mutation is apparently secreted into plasma, but at a significantly reduced level (Figs 2 and 3, top right).

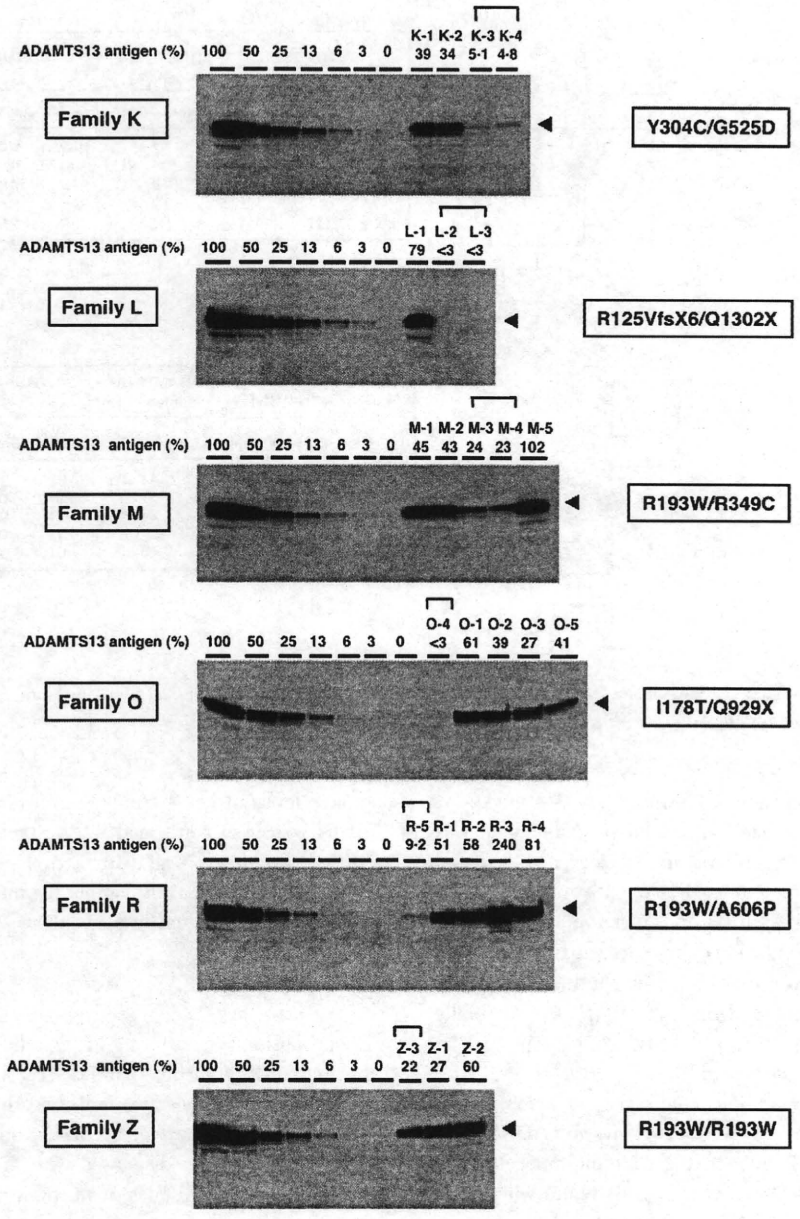
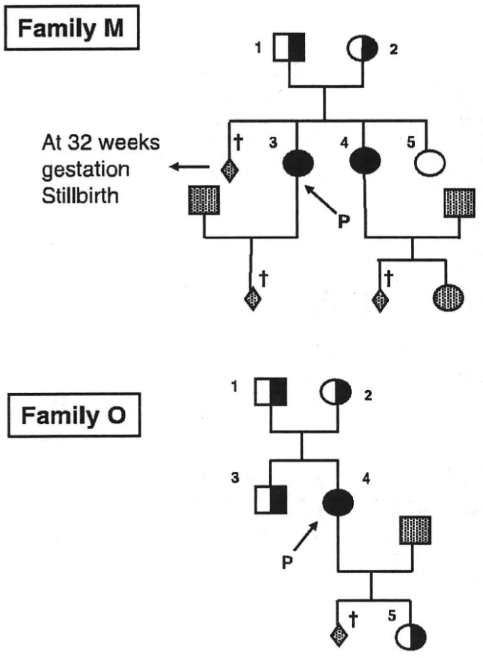



Fig 2. Western blot analyses of ADAMTS13 antigen in plasma milieu of members in USS-Family K~Z. Plasma ADAMTS13 antigen was qualitatively and quantitatively analyzed by Western blot, as described in Materials and methods. Briefly, each 2 μl of diluted or undiluted plasma sample per lane were separated by SDS-5%PAGE under reducing conditions. After electrophoresis, the proteins were blotted onto polyvinylidene difluoride (PVDF) membranes. The ADAMTS13 antigen was incubated with WH2-11-1 as the primary mAb, followed by secondary staining with HRP-conjugated goat anti-mouse IgG, and finally detected by luminography. Densitomeric analyses of ADAMTS13 antigen were performed for the 190-kD band using NIH image J.

*Family O, clinical data*

The proposita O–4, born in 1958, was the second child of two siblings to non-consanguineous parents (Fig 3, bottom). She had no history of severe neonatal jaundice or thrombocytopenia during childhood. At the age of 26 years, she had her first pregnancy, and at 23 weeks gestation she developed thrombocytopenia ($20 \times 10^9$/l) and a diagnosis of ITP was made. At 25 weeks gestation she delivered a premature infant, but it died soon after birth. After delivery, she developed overt TTP, and received plasma exchange for 2 d (one each day). She was diagnosed with chronic relapsing TTP and received




| | ADAMTS13 activity (%) | | ADAMTS13 antigen (%) | *ADAMTS13* gene | | |
|---|---|---|---|---|---|---|
| | act-ELISA | VWFM | ag-ELISA | Arg193 | Arg349 | Haplotype |
| M-1 | 63 | 44 | 57 | R/W | R/R | RR/WR |
| M-2 | 43 | 49 | 63 | R/R | R/C | RR/RC |
| M-3 | <0·5 | <3 | 8·6 | R/W | R/C | WR/RC |
| M-4 | <0·5 | <3 | 7·7 | R/W | R/C | WR/RC |
| M-5 | 127 | 142 | 115 | R/R | R/R | RR/RR |

| | ADAMTS13 activity (%) | | ADAMTS13 antigen (%) | *ADAMTS13* gene | | |
|---|---|---|---|---|---|---|
| | act-ELISA | VWFM | ag-ELISA | Ile178 | Gln929 | Haplotype |
| O-1 | 27 | 18 | 44 | I/T | Q/Q | IQ/TQ |
| O-2 | 26 | 16 | 51 | I/I | Q/X | IQ/IX |
| O-3 | 28 | 26 | 57 | I/I | Q/X | IQ/IX |
| O-4 | <0·5 | <3 | <0·1 | I/T | Q/X | TQ/IX |
| O-5 | 29 | 26 | 65 | I/T | Q/Q | IQ/TQ |

Fig 3. Pedigree and ADAMTS13 analyses of USS-Family M and O. Symbols are the same shown in Fig 1. Mutations found in *ADAMTS13* are shown as one-letter amino acid abbreviations.

prophylactic plasma infusions (400 ml) every 2–4 weeks. At the age of 31 years, she had her second pregnancy and was managed with prophylactic plasma infusions every 1–2 weeks, but at 8 weeks gestation she developed proteinuria and thrombocytopenia ($93 \times 10^9$/l), and therefore she received more frequent plasma infusions (fresh frozen plasma, about 10 ml/kg; 400–480 ml per week) beginning at this time. Under these circumstances, at 39 weeks gestation, she naturally delivered a mature baby who weighed 2·984 kg. The platelet count of this patient decreased to $73 \times 10^9$/l at 7 d after delivery and plasma was administered every 3–4 weeks. In this patient, plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor were assayed in 2004 by the VWF multimer method, showing a severe deficiency of enzyme activity but without an inhibitor, suggesting a diagnosis of USS. In 2005, the act-ELISA revealed that the plasma level of ADAMTS13 activity in this patient was severely deficient (<0·5% of the normal), without an inhibitor (<0·5 BU/ml).

### *ADAMTS13 analysis (heterozygous I178T/Q929X mutation)*

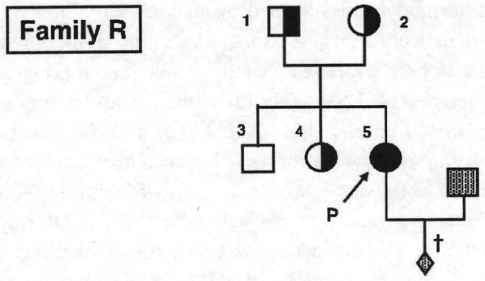
The *ADAMTS13* mutation was the compound heterozygote of I178T (*c.* 533T > C, exon 5) and Q929X (*c.*2785C > T, exon 22) in the proposita (0–4), and her parents, elder brother, and 2nd child (girl) were heterozygote carriers of either of the mutations. One common SNP was found in the patient (Table II).

Plasma level of ADAMTS13 antigen by the ag-ELISA was <0·1% of the normal in the proposita, and ADAMTS13 antigen levels of her 4 family members were 44~65%. These results were also confirmed by Western blot studies (Fig 2). Further, no other band with a molecular weight less than 190 kDa was found. Thus, neither the mutant protein of I178T nor Q929X was present in plasma (Fig 2).

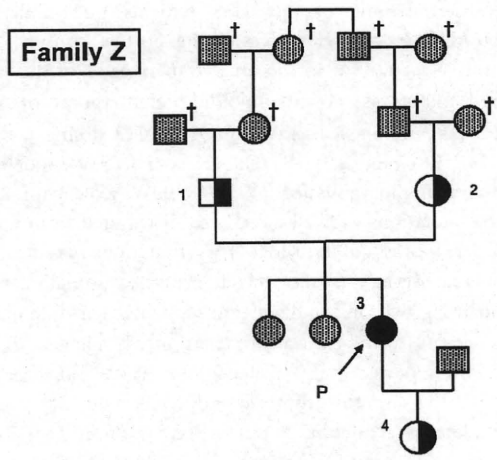
### *Family R, clinical data*

The proposita R-5, born in 1982, was the last child of 3 siblings to non-consanguineous parents (Fig 4, top). She had no history of severe neonatal jaundice. At the age of 8 months she developed generalized petechiae with thrombocytopenia ($20 \times 10^9$/l) and was diagnosed with ITP. Until aged 5 years she had repeated episodes of purpura accompanied by fever that improved spontaneously within a few days. Her two elder siblings were asymptomatic with no history suggesting TTP. At the age of 23 years, she had her first pregnancy. At 23 weeks gestation, she developed mild thrombocytopenia ($82 \times 10^9$/l), which further decreased to $47 \times 10^9$/l at 29 weeks gestation, and was diagnosed with ITP exacerbated by pregnancy. At 30 weeks gestation she had headache and nausea, and a week later she had sudden intrauterine fetal death. After Caesarean section, she developed overt TTP, treated with steroids and plasma exchange, which was performed eight times during the following 23 d. On this occasion in 2005, she was diagnosed with USS after determinations of ADAMTS13 activity (<0·5% of the normal) and ADAMTS13 inhibitor (<0·5 BU/ml) by the act-ELISA. After the diagnosis was confirmed, she has received prophylactic plasma infusions (320 ml every 2 weeks).




| | ADAMTS13 activity (%) | | ADAMTS13 antigen (%) | *ADAMTS13* gene | | |
|---|---|---|---|---|---|---|
| | act-ELISA | VWFM | ag-ELISA | Arg193 | Ala606 | Haplotype |
| R-1 | 22 | 10 | 25 | R/W | A/A | RA/WA |
| R-2 | 25 | 36 | 50 | R/R | A/P | RA/RP |
| R-3 | 117 | 96 | 126 | - | - | - |
| R-4 | 22 | 35 | 66 | R/W | A/A | RA/WA |
| R-5 | <0·5 | <3 | 1·6 | R/W | A/P | WA/RP |




| | ADAMTS13 activity (%) | | ADAMTS13 antigen (%) | *ADAMTS13* gene |
|---|---|---|---|---|
| | act-ELISA | VWFM | ag-ELISA | Arg193 |
| Z-1 | 32 | 29 | 43 | R/W |
| Z-2 | 53 | 58 | 83 | R/W |
| Z-3 | <0·5 | <3 | 11 | W/W |
| Z-4 | 27 | 19 | 17 | R/W |

Fig 4. Pedigree and ADAMTS13 analyses of USS-Family R and Z. Symbols are the same shown in Fig 1. Mutations found in *ADAMTS13* are shown as one-letter amino acid abbreviations.

### *ADAMTS13 analysis (heterozygous R193W/A606P mutation)*

The *ADAMTS13* mutation was the compound heterozygote of R193W (exon 6) and A606P (c.1816G>C, exon 16) in the proposita (R-5), and her parents and elder sister were both heterozygote carriers of either of the mutations. Five common SNPs were found in this patient (Table II).

Plasma level of ADAMTS13 antigen by the ag-ELISA was 1·6% of normal in the proposita; the levels in her three family members were 25~65%. These results were also confirmed by Western blot studies (Fig 2). Thus, the R193W/A606P mutant protein is secreted into plasma, but at a markedly reduced level (Fig 2).

### *Family Z, clinical data*

The proposita Z-3, born in 1971, was the last of three siblings of consanguineous parents; the patient's grandparents were cousins (Fig 4, bottom). She had had neonatal jaundice of moderate severity. She was diagnosed of ITP at the age of 7 years, and had been treated with steroids until 15 years of age. She had her first pregnancy at age 25 years, and at 12 weeks gestation she developed thrombocytopenia ($15 \times 10^9$/l) and was diagnosed with pregnancy-associated ITP. At 32 weeks gestation, she had a live birth by Caesarean section, after which she developed overt TTP, which was treated effectively with daily plasma exchange. This patient was referred to our laboratory in 1998, and plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor were assayed by the VWF multimer method, demonstrating a severe deficiency of enzyme activity but without an inhibitor, suggesting a diagnosis of USS. Prophylactic plasma infusions were not initiated in this patient, and she had more than 5 episodes of TTP during 1998–2005. Each episode was treated with a single plasma infusion (320 ml). In 2006, when she had TTP triggered by a respiratory infection, infusion of plasma induced a severe anaphylactic reaction requiring steroid treatment. The act-ELISA confirmed her plasma level of ADAMTS13 activity was severely deficient (<0·5% of the normal) and ADAMTS13 inhibitor was negative (<0·5 BU/ml).

### *ADAMTS13 analysis (homozygous R193W mutation)*

The *ADAMTS13* mutation was homozygous R193W (exon 6) in the proposita (Z-3), and her parents and child were heterozygote carriers of this mutation. One common SNP was found in this patient (Table II).

The plasma level of ADAMTS13 antigen by the ag-ELISA was 11% of normal in the proposita; the levels in her three

family members were 17~83%. These results were also confirmed by Western blot studies (Fig 2). Thus, the R193W mutation is apparently secreted into plasma, but at a reduced level.

## Discussion

Coombs'-negative haemolysis with neonatal jaundice requiring exchange blood transfusions and thrombocytopenia have been considered to be a clinical hallmark of USS (Fujimura *et al*, 2002). However, only 16 (43%) of our 37 USS patients had episodes of exchange blood transfusion for severe jaundice in this period. Since USS is an autosomal recessive order, the ratio of female-to-male patients should be 1-to-1, but there was female predominance (24-to-13) among our 37 patients. The reason for this apparent gender disparity is probably because many USS patients have a mild disorder and thrombocytopenia is misdiagnosed as ITP. Physicians may overlook this important sign, as in fact 29 (78%) of our 37 USS patients had a history of thrombocytopenia; the remaining 8 were unclear as to whether their platelet count had been checked during their childhood.

Episodes of pregnancy and infections are well known precipitating factors for overt TTP in USS patients, but it is not well understood how often and why TTP episodes are induced by these factors (Weiner, 1987; Vesely *et al*, 2004; Terrell *et al*, 2005; Scully *et al*, 2006). In contrast, a chemical compound, DDAVP (1-deamino-8-D-arginine vasopressin), is well documented to induce TTP in USS patients (Hara *et al*, 1986; Veyradier *et al*, 2006), presumably through a rapid increase of plasma VWF by releasing ultra large(UL)-VWF multimers from vascular endothelial cells, leading to platelet thrombi formation under high shear stress (Yagi *et al*, 2001).

This study has clearly shown that pregnancy consistently induces thrombocytopenia during the second–third trimester, often followed by overt TTP. In normal pregnancies, plasma VWF increases steadily during gestation and reaches a maximum level (200~500% of the normal control) at term with appearance of UL-VWF multimers. The elevated plasma levels of VWF return to normal within one week after delivery (Stirling *et al*, 1984). Thus, the plasma milieu during the third trimester and at delivery appears to be similar to that seen after DDAVP administration. Thus, it is conceivable that the rapidly increased plasma level of UL-VWF multimers plays a critical role in precipitating overt TTP in pregnant women with USS.

More interestingly, however, the onset of clinically overt TTP observed in our USS patients during pregnancy is in contrast to mice with *ADAMTS13* knock-out (Motto *et al*, 2005; Banno *et al*, 2006). As previously shown, the *ADAMTS13* knock-out mice have UL-VWF multimers in their plasma and were potentially thrombogenic in *in vitro* studies using a parallel plate flow-chamber system under high shear stress. However, no thrombotic complications were found in the mice after pregnancy. We assume that it is important to note that haemodynamics formed by anatomically different vasculature networks in these two species significantly differs.

It has been proposed that there may be clinically distinct phenotypes of USS, with early-onset and late-onset types (Furlan & Lämmle, 2001; Camilleri *et al*, 2008). Our experience supports this hypothesis. In fact, three out of the nine women who were diagnosed with USS during pregnancy appeared to lack any clinical signs of TTP during their childhood. The distinction of USS patients into two phenotypes by their plasma levels of ADAMTS13 activity is less likely, because all nine of our USS patients had undetectable ADAMTS13 activity (<0·5% of the normal control) by the sensitive act-ELISA. In the nine women, eight of the *ADAMTS13* mutations were compound heterozygotes of Y304C/G525D (2 siblings), R125VfsX6/Q1302X (2 siblings), R193W/R349C (2 siblings), I178T/Q929X, and R193W/A6060P, and one was homozygous for R193W/R193W. Except for R193W, these mutations were all novel, and distributed from exons 4–28. The location of the *ADAMTS13* mutations in our nine USS patients, seven in the metalloprotease domain and the remaining two on the disintegrin/cysteine-rich domains, may be relevant to the clinical presentation. Camilleri *et al* (2008) recently reported that prevalence of R1060W missense mutation at the seventh thrombospondin-1 domain was associated with late-onset adult TTP, but we have not found the R1060W mutation in our 37 Japanese patients with USS (unpublished). Thus, presently there is no clear evidence dividing USS into two phenotypes on a basis of ADAMTS13 activity levels or *ADAMTS13* mutations. Our observations below also support this concept; two siblings of family M (M-3 and M-4) were the late-onset type, whereas other two siblings of family L (L-2 andL-3) shared two different onset types.

Veyradier *et al* (2003) described that prophylactic plasma infusions (fresh frozen plasma at a single dose of 10 ml/kg) every 3–4 weeks were efficiently performed to prevent periods of TTP in their USS-patients, but more plasma infusions (10 ml/kg during 2–5 consecutive days) were required during each relapse as early as possible. However, a standard therapeutic protocol for plasma infusions for pregnant women with USS has not been established. Our experience here clearly demonstrates that women with USS during pregnancy expose their infants to high risk without prophylactic plasma infusions. The risks for fetal loss are presumably caused by the disturbance of utero-placental circulation with platelet thrombi formed, as shown in Patient K-3 with extensive necrotic lesions in both the placenta and uterus. Among 15 pregnancies (one twin pregnancy), eight infants were stillborn or died soon after birth, and the remaining eight survived but they were premature except for one mature baby (O-5 in Fig 3). Further, seven of eight surviving infants were born by Caesarean section; one with prophylactic plasma infusions, two without plasma infusions, and four with aspirin. Notably, one mature baby (O-5) was born at 39 weeks gestation by natural delivery following plasma infusions to the mother that had started in the very early stage of pregnancy (8 weeks

gestation). This experience clearly indicated that prophylactic plasma infusions (fresh frozen plasma, about 10 ml/kg; 400–480 ml per week) at this stage of pregnancy allowed an uneventful delivery. But the best protocol for plasma infusions to women with USS during pregnancy remains to be determined in future studies.

It is clear that deficient ADAMTS13 activity in maternal plasma is harmful to both the mother and fetus. Retrospectively, we suspect that the first two children from asymptomatic carriers of L-1 (Fig 1, bottom) and M-1/-2 (Fig 3, top) might have had USS, because the former died of intrauterine fetal death at 20 weeks gestation and the latter was a stillbirth at 32 weeks gestation. This speculation is based on the observation of UL-VWF multimers in normal newborn cord blood, despite of subnormal levels of ADAMTS13 activity (Johnson *et al*, 1981; Tsai *et al*, 2002).

Because of these clinical observations, here we strongly recommend that ADAMTS13 activity, and ADAMTS13 inhibitor should be measured in patients with thrombocytopenia during childhood and in association with pregnancy.

## Conflict-of-interest disclosure

YF is a member of clinical advisory boards for Baxter BioScience.

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