The plasma ADAMTS13:AC may decrease in advanced cirrhosis due to reduced ADAMTS13 production in HSCs,[47] enhanced consumption to degrade large quantities of VWF: Ag,[26] and/or its plasma inhibitor.[15,16] We detected plasma ADAMTS13:INH in 83% of the patients with severe to moderate ADAMTS13:AC deficiency, but the ADAMTS13 inhibitory activity was in the marginal zone between 0.5 and 1.0 BU/mL in most cases except one TTP patient (2.0 BU/mL)[33] and one patient with severe ADAMTS13:AC deficiency (3.0 BU/mL).[29] We could detect IgG-type autoantibodies in five end-stage cirrhotic patients with severe ADAMTS13:AC deficiency (<3%).[29] One patient had characteristic clinical features of TTP. However, the remaining four patients did not show any apparent clinical features of TTP, but were indistinguishable from the typical TTP patients based on ADAMTS13:AC and the presence of anti-ADAMTS13 autoantibodies. These results indicate that some end-stage cirrhotic patients with extremely low ADAMTS13:AC and IgG ADAMTS13 inhibitor might have a condition similar to TTP or have "subclinical" TTP. Together with our present results, these findings suggest that the degree of decrease in the plasma ADAMTS13:AC may be a useful predictor that closely correlates with the pathogenesis of hepatic failure, including encephalopathy and/or renal disturbance, in the patients with advanced LC.

Alternatively, various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP.[48] In addition, some patients with congenital TTP whose ADAMTS13:AC is extremely low (<0.5%) due to mutations in the *ADAMTS13* gene showed no apparent clinical features during their childhood except mild to moderate thrombocytopenia during stressful conditions such as infection, but apparently developed "TTP" during the third trimester of pregnancy when VWF production from the placental endothelial cells markedly increases.[49] This condition is called "masqueraded TTP in congenital Upshaw–Schulman syndrome." Furthermore, sepsis-induced multi-organ failure has been recently shown to have close correlation with the decrease in ADAMTS13:AC and increase in VWF: Ag.[50] This finding indicates that the enzyme to substrate ratio is extremely important in the formation of platelet microthrombi and subsequent microcirculatory disturbances that lead to multi-organ failure. In advanced cirrhotics, endotoxemia is frequently detected,[21] and SBP sometimes occurs.[35] HCC becomes highly complicated as the cirrhotic stage progresses,[51] suggesting that these patients are at high risk for platelet microthrombi formation. Therefore, LC patients with moderate ADAMTS13:AC deficiency (3–25%) who have no apparent IgG ADAMTS13 inhibitor and whose inhibitory activity is in the marginal zone (0.5–1.0 BU/mL) may be especially prone to a poorer prognosis in case of infection and endotoxemia that augments VWF production from endothelial cell precipitates, ultimately resulting in a shorter survival.

In summary, the plasma ADAMTS13:AC concomitantly decreased as the functional liver capacity declined, and the degree of activity closely correlated with the prognosis of the LC patients. Therefore, ADAMTS13:AC may be a useful marker to predict the clinical outcome in advanced cirrhosis.

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Reduced larger von Willebrand factor multimers at dawn in OSA plasmas reflect severity of apnoeic episodes

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ABSTRACT: **Plasma von Willebrand factor (VWF), produced in and released from vascular endothelial cells by various stimuli including hypoxia, induces platelet aggregation under high shear stress and plays dual pivotal roles in haemostasis and thrombosis within arterioles, which are regulated by the size of vWF multimers (VWFMs).**

Patients with obstructive sleep apnoea (OSA) have increased risk of thrombotic cardiovascular events, but the pathogenesis is unclear. We examined the relationship between VWF and OSA by measuring VWF antigen (VWF:Ag), VWFMs, VWF collagen binding activity (VWF:CB) and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13. A total of 58 OSA patients were enrolled. Blood samples were collected before sleep, after sleep, and after one night of nasal continuous positive airway pressure therapy.

Based on VWFM analysis, OSA patients were classified into three groups; consistently normal VWFMs (group 1, n=29), increased high molecular weight (HMW)-VWFMs at 06:00 h (group 2, n=18), and decreased or absent HMW-VWFMs at 06:00 h (group 3, n=11). Patients in group 3 had significantly worse apnoea/hypopnoea index; VWF:CB followed a similar pattern. We observed a significant decrease in platelet count between 21:00 h and 06:00 h in OSA patients, potentially associated with reduced larger VWFMs together with decreased VWF:Ag levels. Severe OSA may contribute to an arterial pro-thrombotic state.

KEYWORDS: **ADAMTS13, obstructive sleep apnoea, von Willebrand factor**

Obstructive sleep apnoea (OSA) is characterised by the collapse of the upper airway and associated intermittent hypoxia during sleep [1]. OSA is associated with excessive daytime sleepiness and cardiovascular disease. Patients with OSA often suffer from obesity, hypertension, hyperlipidaemia, and impaired glucose tolerance, and OSA is an independent risk factor for cardiovascular diseases [2–4]. Consistent with this, cardiovascular risk returned to baseline in OSA patients treated with nasal continuous positive airway pressure (CPAP), whereas those with severe untreated OSA maintained a high risk [5]. Recently, some association of OSA with venous thromboembolism in regard to pulmonary embolism has been implicated [6, 7]. However, the mechanism of OSA-associated thrombosis might be multifactorial, and in fact has not been evaluated on a basis of arterial thrombosis, which is generated under high shear stress in microvasculatures, where von Willebrand factor (VWF) plays a critical role as a molecular glue that facilitates platelet aggregation or thrombi.

VWF is a macromolecular plasma protein, which is exclusively produced in and released from vascular endothelial cells, and exerts pivotal effects on both haemostasis and thrombosis. VWF assembles into unusually large VWF multimers (UL-VWFMs) consisting of identical 250 kDa subunits, before its release into the circulation. Under normal circumstances, UL-VWFMs are rapidly cleaved by a specific plasma protease, ADAMTS13 (a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13), under the high shear stress generated in the microvasculature; consequently, VWF circulates in the plasma as a heterogeneous family of multimers ranging in size from 500 to 15,000 kDa. UL-VWFMs play an essential role in primary haemostasis by binding platelets to denuded vascular endothelial tissue. However, in the absence of ADAMTS13 activity (ADAMTS13:AC) due to gene mutation or acquired autoantibodies, UL-VWFMs remain uncleaved and generate platelet hyperaggregation. Uncleaved UL-VWFMs lead to the formation of vast platelet thrombi, known as thrombotic

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TABLE 1 Characteristics of patients with obstructive sleep apnoea (OSA) and sleep controls

	OSA	Sleep controls	p-value
Sex n (M/F)	58 (55/3)	25 (22/3)	NS
Blood type			NS
A	18	12	
B	8	2	
O	26	8	
AB	6	3	
Age yrs	44.7±9.9	38.3±7.1	<0.01
BMI kg·m^{-2}	28.2±3.7	27.7±3.0	NS
AHI	50.5±22.2	4.5±2.8	<0.01
ODI3%	41.6±19.9	7.8±5.1	<0.01
Lowest Sp,O_2 %	76.0±10.0	88.8±5.0	<0.01
Systolic blood pressure mmHg	129±16	122±28	NS
Diastolic blood pressure mmHg	82±12	81±10	NS
vWF:Ag levels % at 06:00 h	103.1±61.4	143.5±63.8	<0.01
ADAMTS13:AC levels % at 06:00 h	56.8±22.6	61.7±20.6	NS

Data are presented as mean±SD, unless otherwise stated. M: males; F: females; BMI: body mass index; AHI: apnoea/hypopnoea index; ODI3%: oxygen desaturation index ⩾3%; Sp,O_2: arterial oxygen saturation measured by pulse oximetry; vWF:Ag: von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; NS: not significant.

thrombocytopenic purpura, a life-threatening generalised disease [8–11].

It is now well established that high plasma levels of VWF antigen (VWF:Ag) are linked with an increased risk for ischaemic heart disease and ischaemic stroke [12–14]. Furthermore, the relative risks of stroke and acute myocardial infarction are higher in individuals with lower ADAMTS13:AC [14, 15]. Furthermore, hypoxia leads to increased VWF release from cultured vascular endothelial cells, both directly, by up regulating VWF expression, and indirectly *via* autocrine and paracrine signalling downstream of hypoxia-induced inflammatory cytokines including interleukin (IL)-6, IL-8, and tumour necrosis factor-α [16, 17]. Despite these important reports of hypoxia-induced VWF secretion, no subsequent studies have addressed the relationship between VWF and the severity of OSA [18, 19]. In particular, no studies have been performed on plasma samples obtained in chronological order relevant to the sleep cycle.

In this study, we sequentially analysed plasma VWF:Ag levels, VWFM patterns, and ADAMTS13:AC in OSA patients not only before and after sleep, but also before and after CPAP treatment. We found that the reduced larger VWFMs together with decreased VWF:Ag levels in the plasma of OSA patients taken at dawn correlate with the clinical severity of apnoeic episodes.

PATIENTS, MATERIALS AND METHODS

Patients

Between February 2004 and April 2011, 284 patients received full standard diagnostic polysomnography (PSG) at Nara Medical University Hospital (Nara, Japan). Among them, 86 patients were diagnosed with normal or mild OSA (apnoea/

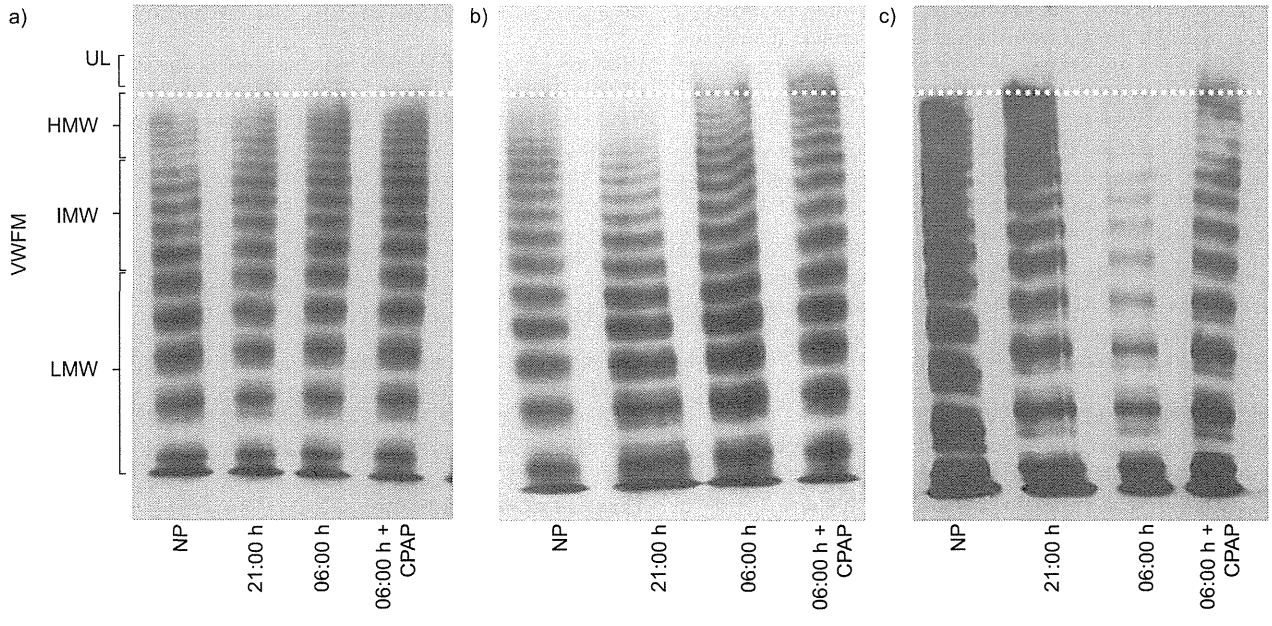

FIGURE 1. Patterns of von Willebrand factor multimers (VWFMs) corresponding to three patient groups. Obstructive sleep apnoea (OSA) patients were categorised into three groups based on the results of VWFM analysis, using sequential samples. Representative results from each group are shown. a) Group 1, patients (n=29) showed a consistently normal pattern of VWFMs. b) Group 2, patients (n=18) had increased, unusually large (UL)- and high molecular weight (HMW)-VWFMs at 06:00 h compared to 21:00 h. c) Group 3,patients (n=11) had decreased UL- and HMW-VWFM at 06:00 h compared to 21:00 h.

hypopnoea index (AHI) <15), and 198 patients were diagnosed with moderate or severe OSA (AHI ⩾15) and received nasal CPAP therapy. Within the latter group, 140 patients with the following underlying diseases were excluded: stroke, coronary artery disease, asthma, chronic obstructive pulmonary disease, arthritis, autoimmune disease, rhinitis, and malignant diseases. The 58 remaining OSA patients were enrolled in this study; detailed clinical information for these 58 patients is shown in table S1. Written informed consent was obtained from all patients, and the study was approved by the Human Subjects Ethics Committee of Nara Medical University (No. 04-012). 25 healthy volunteers (88% male), as shown in table 1, that had undergone PSG studies without OSA were also enrolled and used as the sleep controls.

Blood sampling

Plasma samples were collected from OSA patients at three time points throughout the day; 21:00 h before PSG, at 06:00 h after the PSG without CPAP, and at 06:00 h after CPAP treatment. For the sleep control subjects, plasma samples were collected at 06:00 h. Blood was collected in plastic tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing a tenth volume of 3.8% trisodium citrate an anticoagulant, and platelet-poor plasma was prepared by centrifugation at 3,000 × g for 15 min at 4°C. Aliquots were stored at -80°C prior to use. To obtain platelet counts, blood was collected into plastic whole blood tubes with spray-coated EDTA (Becton, Dickinson and Co.) tubes containing EDTA as an anticoagulant and analysed with a Coulter counter (Beckman Coulter, Tokyo, Japan).

Sleep study

PSG was performed using a computerised polysomnography system (Alice 4; Respironics, Pittsburgh, PA, USA). Data acquisition began at 21:00 h and continued until 06:00 h the following day. Apnoea was defined as a cessation of airflow for ⩾10 s, and hypopnoea was defined as a decrease in airflow at least 50% for a minimum of 10 s or a clear decrease in airflow (⩾20%) followed by either oxygen desaturation ⩾3% or signs of physiological arousal. The AHI was calculated as the number of apnoea/hypopnoea events per hour of total sleeping time. We also calculated the oxygen desaturation index ⩾3% (ODI3%), defined as the number of ⩾3% dips in oxygen saturation per hour of sleep.

During the night, following diagnostic PSG, patients were treated with nasal CPAP (REMstar Auto; Respironics), with PSG monitoring. Apnoeic episodes were substantially reduced or eliminated during treatment with nasal CPAP.

Analyses of VWF:Ag, VWF, and VWF:CB

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, Glostrup, Denmark) [20]. The VWF:Ag level contained in 1 mL of pooled normal human plasma was defined as 100%; VWF:Ag levels in the 20 healthy controls were 102±33% (mean±SD) [21].

VWFMs were analysed by sodium dodecyl sulphate-1.2% agarose gel electrophoresis followed by Western blotting with luminographic detection [22, 23]. The blots were scanned and subjected to densitometric analysis using ImageJ (National Institutes of Health (NIH), Bethesda, MD, USA). Multimers were classified as low molecular weight (LMW-VWFMs; corresponding to bands 1-5 in VWFM analysis), intermediate molecular weight (IMW-VWFMs; bands 6-10), and high molecular weight (HMW-VWFMs; bands ⩾11) [24]. High molecular weight bands that were not detected in normal plasma (NP) were defined as UL-VWFMs. The levels of LMW-, IMW- and HMW-VWFMs were calculated using NIH ImageJ. For quantitative analyses, we calculated the ratios of the densities of VWFMs, LMW, IMW, and HMW relative to total VWFM density. Further, multimeric VWF:Ag levels were calculated by multiplying VWF:Ag level by the LMW, IMW, and HMW ratios.

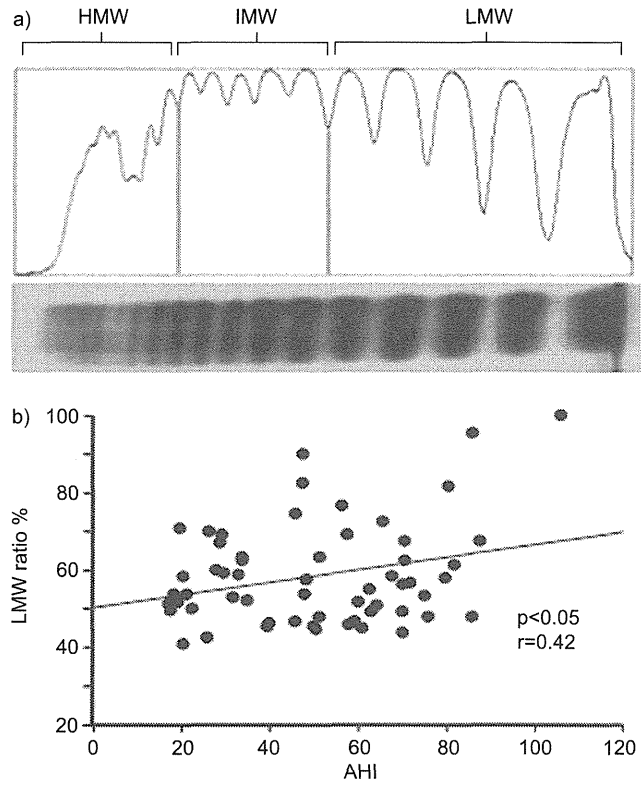

FIGURE 2. Relationship between low molecular weight (LMW) von Willebrand factor multimers (VWFMs) to total VWFMs (LMW ratio) and hypoxia. a) Quantitative analysis of VWFMs was performed by calculating the density of LMW-VWFMs relative to total M density. A representative result of VWF analysis at 06:00 h is shown. b) The LMW ratio of obstructive sleep apnoea patients was significantly correlated to apnoea/hypopnea index (AHI). IMW: intermediate molecular weight.

The plasma VWF collagen binding activity (VWF:CB) was measured using an enzyme immunoassay using a commercially available kit (VWF-CBA ELISA, PROGEN Biotechnik GmbH, Heidelberg, Germany) according to the manufacturer's instructions.

Assay of ADAMTS13:AC

ADAMTS13:AC was determined using a commercially available chromogenic ELISA/ACT (Kainos Co., Tokyo, Japan). The detection limit of this assay was 0.5%; the values obtained from 55 healthy controls were 99.1±21.5% (mean±SD) [25].

Statistical analysis

Laboratory data are expressed as the mean±SD. Comparisons between OSA patients and controls were analysed using the

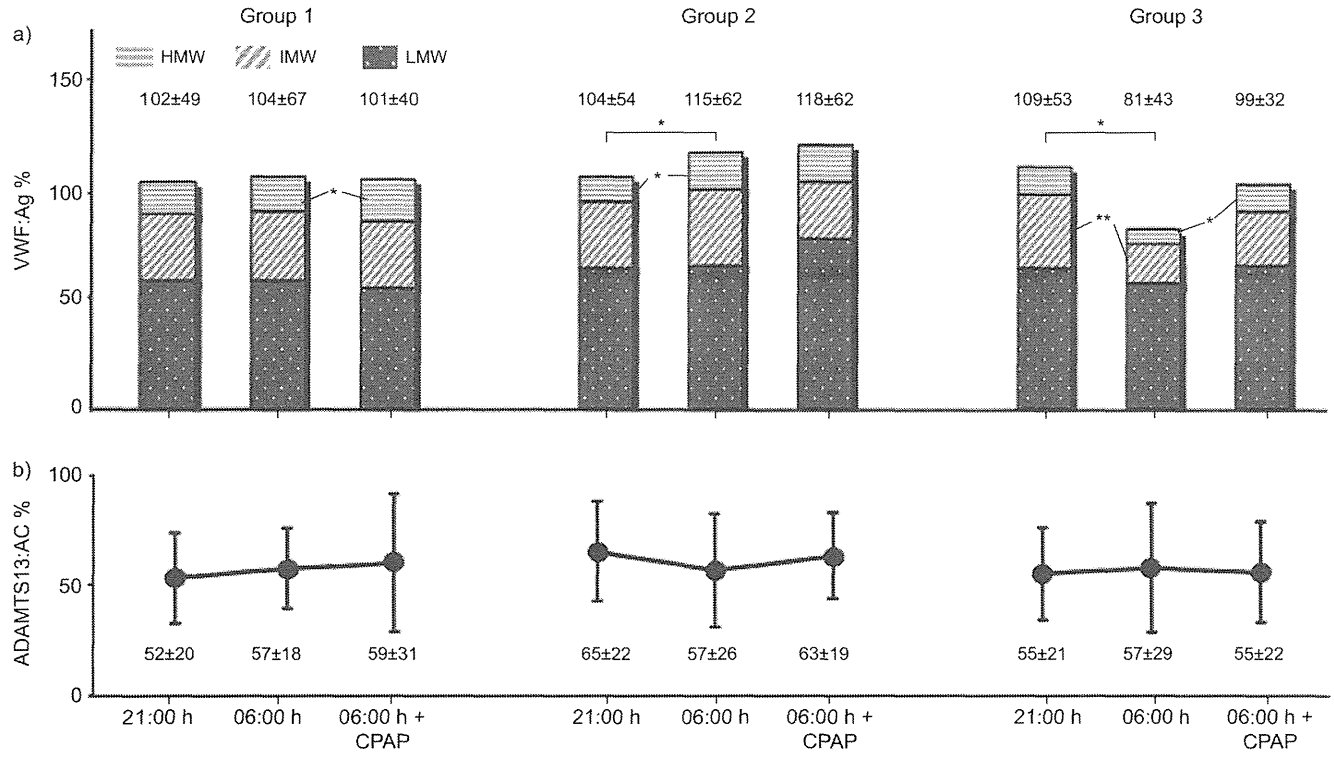

FIGURE 3. Changes in serial von Willebrand factor antigen (VWF:Ag) levels and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity (ADAMTS13:AC) in groups 1–3. VWF:Ag levels were divided into high molecular weight (HMW)-, intermediate molecular weight (IMW)-, and low molecular weight (LMW)-VWFM groups by multiplying the VWF:Ag level by the results of the multimeric analyses. Data are presented as mean±SD . Groups were first compared using the Kruskal-Wallis H-test; significantly different groups were then analysed using the Mann-Whitney U-test *: $p<0.05$; **: $p<0.01$.

Mann-Whitney U-test or Chi-square test. All comparisons among the three groups were tested for statistical significance using the Kruskal-Wallis H-test or Chi-square test, with Yates' correction for 2×3 tables; significant differences between the three groups (overall $p<0.05$) were further analysed using the Mann-Whitney U-test or Chi-square test. All analyses were carried out using StatView (SAS Institute Inc., Cary, NC, USA). A p-value <0.05 was considered significant.

RESULTS

Characteristics of patients with OSA and controls

The demographics and sleep characteristics of patients with OSA and controls are shown in table 1. Patients with OSA were slightly older than the control population but were otherwise similar demographically. 18, seven, and four patients in the OSA group were being treated for hypertension, hyperlipidaemia, and diabetes mellitus, respectively, but no diabetic patients were receiving insulin therapy. Based on the PSG results, the two populations differed significantly with respect to AHI, ODI3%, and lowest S_{p,O_2} %.

Plasma VWF:Ag levels at 06:00 h were significantly lower in patients with OSA compared with the controls, but plasma ADAMTS13:AC at 06:00 h did not differ between these groups. Interestingly, the plasma ADAMTS13:AC at 06:00 h in both OSA patients and sleep controls were lower than those of the above mentioned healthy controls ($p<0.01$).

Chronological changes of plasma VWFM patterns categorise the patients with OSA into three groups

We analysed VWFM patterns in plasmas taken from OSA patients, obtained at 21:00 h and 06:00 h following sleep with or without CPAP. Based on these results, we categorised the patients with OSA into three groups (fig. 1). Patients in group 1 ($n=29$) had a consistently normal pattern of VWFM, almost indistinguishable from that of the sleep controls ($n=6$). Patients in group 2 ($n=18$) exhibited reduced HMW-VWFMs at 21:00 h and persistent UL-VWFMs at 06:00 h, with or without CPAP. Patients in group 3 ($n=11$) had normal VWFM patterns at 21:00 h, reduced predominantly HMW-VWFMs at 06:00 h without CPAP, and returned to a normal VWFM pattern after CPAP therapy.

The decrease in HMW-VWFMs and concomitant increase in LMW-VWFMs could reflect either enhanced proteolysis by ADAMTS13 or extensive consumption secondary to platelet aggregation. Therefore, we first calculated the ratio of LMW-VWFMs to total VWFMs (LMW ratio) at 06:00 h without CPAP (fig. 2), and subsequently determined the relationship between LMW ratio and AHI. As shown in figure 2, these two parameters are significantly correlated ($p<0.05$), suggesting that the

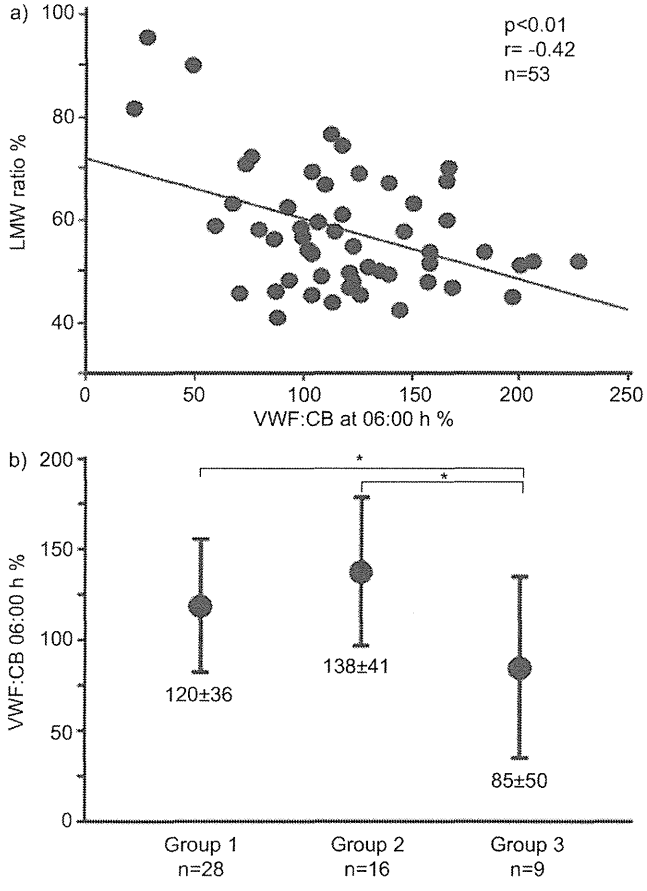

FIGURE 4. Relationship von Willebrand factor (VWF) collagen binding activity (VWF:CB) and ratio of low molecular weight (LMW)-VWF multimers (Ms) to total VWFMs (LMW ratio) and comparison of vWF:CB at 06:00 h in each group. vWF:CB was measured in 53 out of 58 obstructive sleep apnoea (OSA) patients. a. Significant inverse correlation between LMW ratio and VWF:CB at 06:00 h in OSA patients. b) VWF:CB at 06:00 h in group 3 was significantly lower than in groups 1 and 2. Data are presented as mean±SD *: $p<0.05$.

degree of hypoxia during apnoeic events is related to vFWMs processing and/or consumption.

Chronological changes of plasma levels of VWF:Ag, VWFM ratio, and ADAMTS13:AC in three patient groups with OSA

Plasma levels of VWF:Ag at 21:00 h, 06:00 h without CPAP, and 06:00 h with CPAP were determined in all three groups of OSA patients. As shown in figure 3, plasma VWF:Ag levels were almost unchanged in group 1 patients, but significantly increased between 21:00 h and 06:00 h in group 2 patients. Notably, VWF:Ag levels remarkably decreased between 21:00 h and 06:00 h in group 3.

We then determined levels of HMW, IMW, and LMW in all three groups. In group 1, HMW-VWFM showed a slight increase at 06:00 h with CPAP, relative to 06:00 h without CPAP. In group 2, HMW-VWFMs significantly increased at 06:00 h compared to 21:00 h confirming the results of the VWFM analysis used for defining groups 1–3. Consistent with this, in group 3, the IMW-VWFMs at 06:00 h was significantly lower than that at 21:00 h; CPAP treatment reversibly increased the HMW-VWFM at 06:00 h, in accordance with the increase in plasma VWF:Ag level.

In contrast, no change in the plasma ADAMTS13:AC was seen at 21:00 h, 06:00 h, or 06:00 h with CPAP in any of the three groups. These data argue that consumption of the HMW-VWFMs occurred overnight in OSA patients.

Plasma levels of VWF:CB activity

We observed dynamic chronological changes in plasma VWF:Ag levels and VWFM patterns in our subjects, especially in group 3. VWF:CB represents a biological function of VWF, in which HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. In this study, we were able to examine plasma VWF:CB levels in 53 out of 58 OSA patients. As expected, plasma levels of VWF:CB at 06:00 h without CPAP were inversely correlated with the LMW ratio ($p<0.01$), as shown in figure 4. Furthermore, as shown in figure 4, plasma levels of VWF:CB at 06:00 h was significantly lower in group 3 (85±50%) than in either group 1 (120±36%) or group 2 (138±41%). These results argue that structurally and functionally impaired VWFMs were present at 06:00 h in group 3 patients.

Decreased platelet counts at dawn in the untreated patients with OSA

A pair of platelet counts at 21:00 h and 06:00 h without CPAP was determined in 31 of the 58 OSA patients and in six of the 25 sleep controls, all of whom were involved in the later phase of this study. To correct for a possible hydration effect during sleep, we calculated the ratio of platelet count to haematocrit. The ratios in sleep controls did not exhibit significant changes between 21:00 h and 06:00 h (fig. 5), whereas they were lower at 06:00 h in untreated OSA patients ($p<0.01$) (fig. 5). However, none of the patients who received CPAP treatment developed overt clinical signs of thrombotic complications. These results suggest that platelet consumption, to a lesser extent, might occur during sleep without distinct thrombotic symptoms in untreated OSA patients.

Patient characteristics of groups 1, 2, and 3

Table 2 summarises the demographic and measured parameters of OSA patients categorised into groups 1–3. These three groups did not differ demographically, but AHI was significantly higher in group 3 than in groups 1 and 2. ODI3% in group 3 was also significantly higher than in group 1. These results unambiguously indicate that patients in group 3, who exhibit lower levels of large VWFM at 06:00 h represent the highest severity of OSA among the three groups.

Consistent with these results, decreased plasma levels of VWF:Ag in the two different time intervals (06:00 h and 21:00 h) was remarkable in group 3, in comparison to those in groups 1 and 2. Interestingly, the differences of LMW ratio in the two times (06:00 h and 21:00 h) was significantly higher in group 3 than those of groups 1 or 2. These results indicated that decreased VWF:Ag at 06:00 h was caused primarily by the reduction in larger VWFMs. Alternatively, no significant change in ADAMTS13:AC between the two times (06:00 h and 21:00 h) was observed in group 3, whereas such a change was observed in groups 1 and 2, leaving the physiological relevance unaddressed.

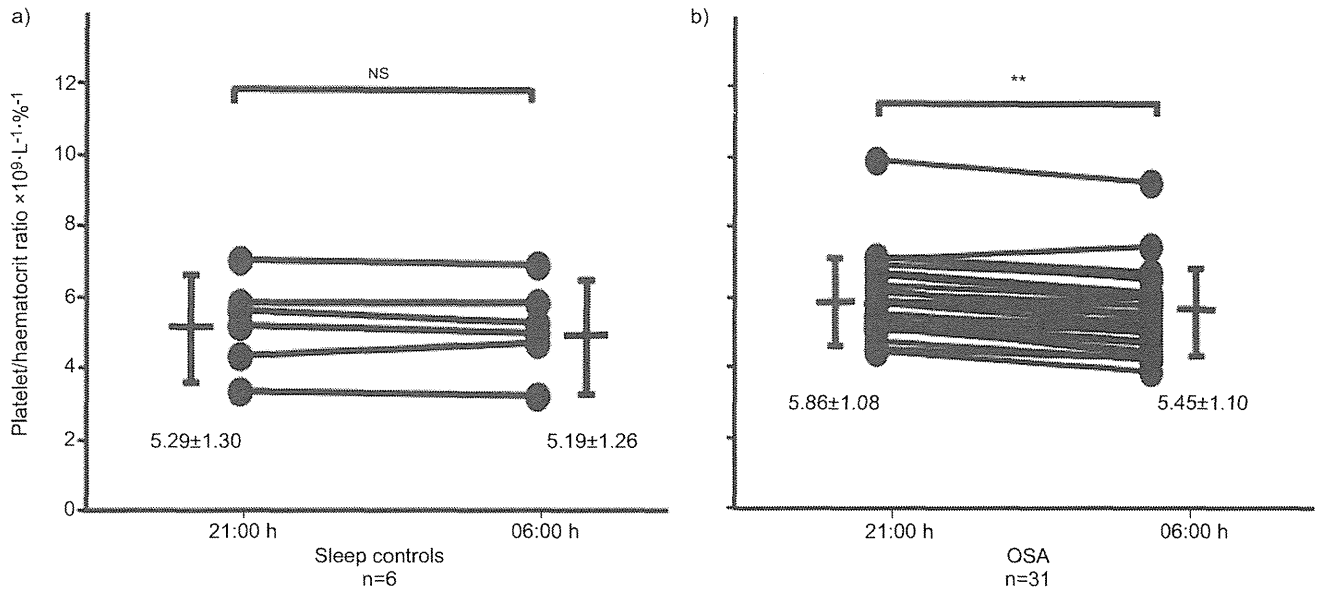

FIGURE 5. Overnight platelet counts to haematocrit ratios decreased in patients with obstructive sleep apnoea (OSA). Platelet counts were normalised to the patient's haematocrit to control for differences in hydration status. Ratios of platelet count to haematocrit were obtained at 21:00 h and 06:00 h in a) six sleep controls and in b) 31 OSA patients, both without CPAP treatment. In the sleep controls, the ratios did not change between time points. In the OSA patients, the ratio exhibited significant changes between time points. Data are presented as mean±SD. NS: nonsignificant. **: $p<0.01$.

Relationship of AHI and groups 1–3 of VWFM patterns in OSA patients

AHI is an excellent means of showing OSA severity, here we have used it to categorise three groups: moderate ($15 \leqslant AHI<30$), severe ($30 \leqslant AHI<60$), and extremely severe ($AHI \geqslant 60$). As shown in table 3, OSA patients with group 1 and 2 consisted of those with variable AHI levels. Notably, none of the OSA patients within group 3 had an AHI 15~<30, and they uniformly

TABLE 2 Characteristics and different parameter between 21:00 h and 06:00 h of patients with obstructive sleep apnoea (OSA) in groups 1–3

	Group			Overall p-value
	1	2	3	
Sex M/F	28/1	18/0	9/2	NS
Blood type				NS
A	7	4	7	
B	4	4	0	
O	14	7	4	
AB	4	3	0	
Age yrs	46.0±9.6	42.9±9.7	44.2±11.3	NS
AHI	43.1±20.0	51.4±19.6	68.7±22.6	<0.05*
ODI3%	35.7±18.2	44.1±19.3	53.2±21.5	<0.01#
Differences in time intervals 06:00 and 21:00 h				
VWF:Ag %	2.1±34.8	10.8±22.0	-28.1±40.6	<0.05¶
LMW ratio %	-0.27±5.24	-4.46±8.69	16.69±16.92	<0.01¶
ADAMTS13:AC %	4.4±13.1	-8.5±25.9	2.4±21.4	<0.05+
Plt/Ht ×10^9·L^{-1}·$\%^{-1}$	-0.045±0.036 (n=15)	-0.034±0.038 (n=10)	-0.043±0.029 (n=6)	NS

Data are presented as n or mean±SD, unless otherwise stated. M: males; F: females; AHI : apnoea/hypopnea index, ODI3%: oxygen desaturation index ⩾3%; vWF:Ag : von Willebrand factor antigen; LMW ratio: the ratio of low molecular weight-VWFMs to total VWFMs; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; Plt/Ht: platelet count to haematocrit ratio. NS: not significant. *: $p<0.05$ between groups 1, 2 and 3; #: $p<0.01$ between groups 1 and 3; ¶: $p<0.01$ between groups 1, 2 and 3; +: $p<0.05$ between group 1 and 2.

TABLE 3 Characteristics and thrombotic parameters of patients classified with apnoea/hypopnoea index (AHI)

	15≤AHI<30	30≤AHI<60	AHI≥60	Overall p-value
Patients n	15	22	21	
Sex M/F	15/0	21/1	19/2	NS
Age yr	43.7±12.0	42.9±9.7	44.2±11.3	NS
ODI3%	19.2±4.9	36.2±10.9	63.3±9.4	<0.01**
VWFM group				
1	12 (80)	8 (36)	9 (43)	<0.05*
2	3 (20)	10 (45)	5 (24)	NS
3	0	4 (18)	7 (33)	<0.05#
VWF:Ag at 06:00 h %	98.5±49.1	98.5±55.7	111.3±75.5	NS
ADAMTS13:AC at 06:00 h %	58.1±20.2	55.2±21.9	57.6±25.6	NS
VWF:CB at 06:00 h U·mL^{-1}	1.29±0.39 (n=13)	1.23±0.50 (n=19)	1.09±0.38 (n=19)	NS
Plt/Ht at 06:00 h ×10^{9}·L^{-1}·%$^{-1}$	0.526±0.093 (n=10)	0.549±0.138 (n=13)	0.561±0.087 (n=8)	NS

Data are presented as mean±SD or n (%), unless otherwise stated. M: males; F: females; ODI3%: oxygen desaturation index ≥3%; VWFM: von Willebrand factor multimer; VWF:Ag : von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; Plt/Ht: platelet count to haematocrit ratio; NS: not significant. *: p<0.05 between 15≤AHI<30 and 30≤AHI<60, AHI≥60, **: p<0.01 between all AHI groups; #: p<0.05 between 15≤AHI<30 and AHI≥60.

had AHI ≥30 and more predominantly with AHI ≥60. The incident for group 1 patients was lower in AHI groups of 30≤AHI<60 and AHI≥60 than those of 15≤AHI<30 (p<0.05). In contrast, the incident for group 3 was higher in AHI≥60 than those of 15≤AHI<30 (p <0.05). No significant relationship between AHI score and each parameter such as VWF, ADAMTS13, or platelet count was found.

DISCUSSION

Plasma VWF:Ag levels increase after the age of 40 yrs in normal individuals; by the age of 60 yrs they can have reached ~120–140% of the healthy normal baseline [26]. The mean age of OSA patients enrolled in this study was 44.7 yrs, whereas that of control subjects was 38.3 yrs. However, the plasma VWF:Ag levels collected at 06:00 h were significantly lower for OSA patients than for control subjects (table 1). In contrast, plasma ADAMTS13 activity decreases after the age of 40 yrs in normal individuals [27]. Among our study patients and controls, plasma ADAMTS13:AC was lower than in healthy controls aged between 20-40 yrs (p<0.01), indicating that these two groups did not significantly differ (table 1).

Given the observed differences in VWF:Ag levels between OSA patients and control subjects, we analysed VWFM patterns chronologically at three time points: at 21:00 h and at 06:00 h either with or without overnight CPAP treatment. As expected, a majority of OSA patients (29 (50%) out of 58) had consistently normal VWFM patterns, categorised as group 1. Two smaller groups of patients had increased UL- and HMW-VWFM (18 (31%) out of 58) or decreased UL- and HMW-VWFM (11 (19%) out of 58) at 06:00 h; these were categorised as group 2 or group 3, respectively. The ratio of LMW-VWFM to total VWFM, termed the LMW ratio, is a determination of the relative amount of degraded VWFM; in our study population, the LMW ratio correlated significantly with the AHI.

The increased LMW ratio seen in OSA patients could arise from reduced production of VWF by vascular endothelial cells, increased clearance of HMW-VWFM from the circulation, or consumption during thrombosis. However, *in vitro* studies have clearly shown that VWF expression by cultured vascular endothelial cells is increased under conditions of hypoxia; it is unlikely that patients with OSA, a condition of intermittent hypoxia, would exhibit decreased expression of VWF overnight [17]. Additionally, no differences were seen in the plasma ADAMTS13:AC in any group at any time-point, suggesting that enhanced proteolysis of HMW-VWFM was not occurring. Therefore, we hypothesised that the elevated LMW ratio seen in our OSA patients was likely to be due to an enhanced degradation or consumption of HMW-VWFM.

The cause of thrombotic complications in OSA patients might be multifactorial, but in this study we have clearly indicated that VWF appears to play an essential role in the thrombogenesis in a certain population categorised as group 3. Although the mechanism is not yet fully elucidated, the high VWFMs released upon hypoxia from vascular endothelial cells is a most plausible factor. Thus, severe OSA could be a risk factor for both arterial and venous thrombosis as described in the introduction.

To better understand whether some degree of thrombosis was occurring overnight in untreated OSA patients, we determined platelet counts in 31 out of 58 patients; we observed a significant decrease in platelet count between 21:00 h and 06:00 h. This decrease was associated with reductions in both the plasma VWF:Ag levels and HMW-VWFM in group 3. Quantitative analyses of VWFMs in group 3 showed that levels of HMW-VWFMs increased significantly after CPAP treatment, compared with measurements taken at 06:00 h without CPAP. This is consistent with low-level consumption of UL- and HMW-VWFMs by microvascular thrombus formation and/or platelet aggregation during sleep in OSA patients; CPAP therapy might reduce such consumption. However, no patients have developed overt clinical signs of thromboembolic complications; therefore, we prefer to use the term "pre-clinical platelet consumption"

to describe this phenomenon. This may represent a baseline pro-thrombotic state in OSA patients that can be corrected by CPAP therapy.

In this study, the chronological analyses have unanimously indicated that reduced large VWFMs in plasmas at dawn reflect the clinical severity of apnoea in OSA patients. The results obtained by VWFM analysis were solid, but the procedure was time consuming and requires a high technical skill to perform. A reliable high-throughput method would be necessary for routine clinical use. In this regard, the assay for VWF:CB is a promising candidate for such a method, because HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. Our results indicated that VWF:CB at 06:00 h correlated well with VWFM patterns, and was consistent with earlier assignment of subjects to groups 1–3. Thus, through this study we have provided the first convincing evidence that VWF at dawn in group 3 was impaired not only structurally but also functionally, presumably due to hypoxia-induced release and consumption of VWF. This process might also involve platelet aggregation and consumption, even though the patients were asymptomatic. Thus, large scale studies, together with chronological measurements of platelet counts and VWF:CB, would be the focus in the following studies.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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Acquired Idiopathic ADAMTS13 Activity Deficient Thrombotic Thrombocytopenic Purpura in a Population from Japan

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Abstract

Thrombotic thrombocytopenic purpura (TTP) is a type of thrombotic microangiopathy (TMA). Studies report that the majority of TTP patients present with a deficiency of ADAMTS13 activity. In a database of TMA patients in Japan identified between 1998 and 2008, 186 patients with first onset of acquired idiopathic (ai) ADAMTS13-deficient TTP (ADAMTS13 activity <5%) were diagnosed. The median age of onset of TTP in this group of patients was 54 years, 54.8% were female, 75.8% had renal involvement, 79.0% had neurologic symptoms, and 97.8% had detectable inhibitors to ADAMTS13 activity. Younger patients were less likely to present with renal or neurologic dysfunction ($p<0.01$), while older patients were more likely to die during the TTP hospitalization ($p<0.05$). Findings from this cohort in Japan differ from those reported previously from the United States, Europe, and Korea with respect to age at onset (two decades younger in the other cohort) and gender composition (60% to 100% female in the other cohort). We conclude that in one of the largest cohorts of ai-TTP with severe deficiency of ADAMTS13 activity reported to date, demographic characteristics differ in Japanese patients relative to those reported from a large Caucasian registry from Western societies. Additional studies exploring these findings are needed.

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disorder and originally defined by classic "pentad"; thrombocytopenia, microangiopthic hemolytic anemia (MAHA), renal impairment, neurological symptoms, and fever [1]. In 1998, two studies identified deficiency of plasma ADAMTS13 (a disintegrin-like and metalloprotease with thrombospndin type 1 motifs 13) activity (ADAMTS13:AC) among persons with TTP [2,3]. ADAMTS13 cleaves the peptide bond between Thy1605 and Met1606 in the A2 domain of von Willebrand factor (VWF) subunit. VWF is synthesized in vascular endothelial cells and megakaryocytes. Vascular endothelial cell-derived VWF is released into the plasma as unusually large VWF multimers (UL-VWFMs). UL-VWFMs are degraded into smaller size VWF multimers by ADAMTS13. Severe deficiency of ADAMTS13:AC, either congenital or acquired, results in accumulation of UL-VWFMs and formation of platelet thrombi in the microvasculatures. In congenital TTP (Upshaw-Schulman syndrome), ADAMTS13 deficiency is caused by mutations in the ADAMTS13 gene [4]. In contrast, acquired TTP is frequently caused by inhibitory autoantibodies against ADAMTS13 [2,3]. Most acquired TTP patients have IgG antibodies. In rare cases, IgA and/or IgM antibodies are associated with IgG antibodies [5,6]. Patients with severe ADAMTS13:AC deficiency present with a lower platelet count and a significantly increased risk of TTP relapse [7–10]. Only a few small cohort studies of acquired idiopathic TTP patients characterized by severe ADAMTS13:AC deficiency have been reported previously. These studies characterize TTP with a predilection for the young and female, high rates of renal and central nervous system (CNS) involvement, and a 15% to 20% mortality. The largest cohort of acquired idiopathic (ai)-severely ADAMTS13-deficient TTP patients previously reported is from the Oklahoma TTP Registry (n = 60) [10]. In this study we systematically analyzed the clinical and laboratory features of a large cohort of Japanese patients with acquired idiopathic TTP and who also have severe ADAMTS13:AC deficiency.

Results

The number of ai-TTP patients fit the above inclusion criteria and retained for the study was 186. Of these, 31 (16.7%) were diagnosed between 1998 and 2001, 84 (45.2%) between 2002 and

2005, and 71 (38.2%) since 2006. This included individuals who did not experience any exposure to drugs that cause TTP or TMA, organ transplantation, stem cell transplantation, immunologic disease and also did not have a prior history of TTP. The age distribution of disease onset ranged from 8 months to 87 years old, with peak incidence occurring at age 60 (Figure 1, upper panel). Patients under 20 years accounted for 9.1% (17/186) of this subgroup, while patients over age 80 years accounted for 3.8% (7/186). Females accounted for 54.8%. Laboratory studies revealed that 100% of these patients were thrombocytopenic, 75.8% had renal involvement, and 79.0% had neurologic involvement. Overall, 16.1% died from TTP. ADAMTS13 inhibitors (≥0.5 BU/ml) were identified in 182 patients (97.8%). As shown in Figure 1 lower panel, 8.1% of these patients had inhibitor titers of 0.5~<1.0 BU/ml, 35.5% had titers of 1.0~<2.0, 33.3% had inhibitor titers of 2.0~<5.0, 12.9% had inhibitor titers of 5.0~<10, and 8.1% had inhibitor titers of ≥10 BU/ml. We found four ai-TTP patients without ADAMTS13 inhibitor (<0.5 BU/ml), whose ADAMTS13:AC, however, was normalized after remission. Therefore, these patients were included in this study.

The ai-TTP patients were evaluated according to the age at diagnosis (Table 1); Group 1 (<20 years old: n = 17), Group 2 (20~<40 years old: n = 36), Group 3 (40~<60 years old: n = 63), and Group 4 (60 years old~: n = 70). Rates of renal and neurologic dysfunction at the time of TTP presentation were lowest in the youngest age-subgroup (52.9% versus 72.2% to 81.0% for renal involvement, and 47.0% versus 69.4% to 88.6% for neurologic involvement; $p<0.01$) while in-patient mortality was highest among the oldest sub-group (28.6% versus 5.9% to 11.1%, $p<0.01$). Overall, females accounted for 54.8% of the patients (with rates of female gender ranging from 45.7% to 69.4% in each of the four age-groups).

Discussion

We evaluated 186 patients with initial onset of severely deficient ADAMTS13:AC levels TTP in Japan, representing the largest cohort of ai-TTP patients with ADAMTS13:AC deficiency reported from Japan. These individuals had presented with TMA-findings to medical centers throughout Japan over a ten-year period. In interpreting our findings, several factors should be considered.

These individuals accounted for 71.5% of 260 patients with a first episode of ai-TTP, who were diagnosed in out registry. This rate is similar to that reported previously for smaller cohorts of TTP patients from Europe, the United States, Canada, the United Kingdom, and Korea [7–13].

Sociodemographic characteristics of these TTP patients were compared to those reported from cohorts in Oklahoma, Saint Louis, France, and Korea [7–10,13] (Table 2). The median age of TTP patients with severely deficient ADAMTS13:AC levels reported from the other cohorts except for Saint Louis is 15 to 20 years less than that reported for our cohort. Also, females accounted for 53.8% of patients in our cohort versus 60% to 100% in other cohorts. Since additional information on predisposing factors for TTP are not known currently, it is not possible to identify factors accounting for age- and gender-related differences noted between TTP patients in Japan with severe ADAMTS13-deficiency versus those reported from other geographic regions.

Age-related differences in rates of neurologic and renal involvement among TTP patients who had severely deficient ADAMTS13:AC levels have not been reported previously. We found lower rates of renal and neurologic dysfunction amongst the youngest TTP patients, and the highest short-term mortality rates among the oldest TTP patients. While our study evaluated 186 patients with initial onset of severe ADAMTS13:AC activity

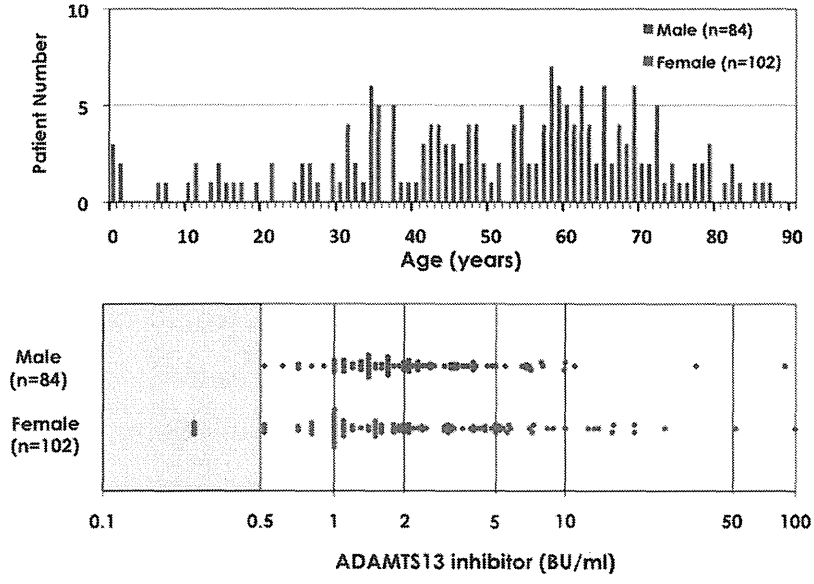

Figure 1. Age distribution and ADAMTS13 inhibitor levels in acquired idiopathic (ai−) TTP with severe deficiency of ADAMTS13 activity. Upper panel shows the age distribution of 186 patients with severe deficiency of ADAMTS13 activity under 5%. We found wide range of the age at TTP bouts from 8 months old to 87 years old. The highest incident peak was found around 60 years old. Lower panel shows the distribution of ADAMTS13 inhibitors in 186 ai-TTP patients with severe deficiency of ADAMTS13 activity. We found ADAMTS13 inhibitors (≥0.5 BU/ml) in 182 patients (97.8%). High titer inhibitors ≥2.0 BU/ml was seen in 101 patients (54.3%).
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Table 1. Clinical features in ai-TTP patients with severe deficiency of ADAMTS13:AC.

	All patients	Groups according to age				
		1	2	3	4	Overall p
Age (years)	54 (37, 65)	<20	20~<40	40~<60	60~	
	Median (25, 75 percentile)					
Patient Number	186	17	36	63	70	
Female (%)	54.8	52.9	69.4	57.1	45.7	NS
"Pentad"						
(1) Platelet count ($\times 10^9$/L), Median (25, 75 percentile)	10 (7, 16)	9 (7, 12)	10 (7, 20)	10 (6, 18)	10 (8, 15)	NS
(2) Hemoglobin (g/dL), Median (25, 75 pecentile)	7.3 (6.1, 8.7)	7.4 (5.4, 8.7)	6.7 (5.9, 7.8)	7.1 (6.0, 8.8)	7.8 (6.6, 8.8)	NS
(3) Renal involvement (%)	75.8	52.9	72.2	81.0	78.5	NS
Serum creatinine (mg/dL), Median (25, 75 percntile)	0.9 (0.7, 1.3)	0.58 (0.31, 0.80)	0.86 (0.70, 1.16)	0.95 (0.80, 1.50)	1.00 (0.80, 1.40)	<0.01[a]
Blood urea nitrogen (mg/dL), Median (25, 75 percntile)	24 (17, 37)	15 (12, 23)	19 (14, 26)	27 (17, 41)	27 (21, 43)	<0.01[b]
(4) CNS involvement (%)	79.0	47.0	69.4	82.5	88.6	<0.01[c]
(5) Fever (≥37.0°C) (%)	71.5	76.5	63.9	69.8	75.7	NS
Mortality in the current episode of TTP bouts (%)	16.1	5.9	5.6	11.1	28.6	<0.05[d]

NS: not significant difference (≥0.05).
Overall p values were caluculated using the Kruskal-Wallis H tests or chi-square tests with Yates' correction for 2×4 tables.
Significant differnces between 4 groups (overall p<0.05) were further analyzed by Mann-Whitney U-test or chi-squre test.
[a]p<0.01 between Group 1 and Groups 2, 3, 4.
[b]p<0.01 between Group 1 and Groups 3, 4, and between Group 2 and Groups 3, 4.
[c]p<0.01 between Group 1 and Groups 3, 4.
[d]p<0.05 between Group 2 and Group 4.
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Table 2. Comparison of our findings with those reported from Europe, Asia, and the United States for acquired idiopathic TTP patients with severely deficient ADAMTS13:AC levels.

	This study	Vesely et al[7]	Zheng et al[8]	Coppo et al[9]	Kremer-Hovinga et al[10]	Jang et al[13]
	(n = 186)	(n = 16)	(n = 16)	(n = 31)	(n = 60)	(n = 20)
Geographic region	Japan	Oklahoma (USA)	Saint Louis (USA)	France	Oklahoma (USA)	Korea
Ethnicity/race	Japanese 100%	White 50%, African-American 50%	White 32%, African-American 68%	White 52%, Afro-Caribbean 48%	African-American 35%	Korean 100%
Idiopathic etiology	100%	100%	100%	100%	77%	70%
Prior TMA	0%	0%	38%	13%	0%	ND
ADAMTS13:AC	<5%	<5%	<5%	<5%	<10%	<10%
ADAMTS13:INH	98%	94%	44%	55%	83%	ND
Age (years)	54 (8 m–87)	39 (19–71)	51 (21–79)	36 (19–67)	41 (9–72)	40.5 (mean)
% female	55	75	100	65	82	60
Platelets (10^9/ul)	10 (1–88)	11(4–27)	17 (6–47)	12 (2–69)	11 (2–101)	24 (mean)
Hb (g/dl)	7.3 (4.3–11.9)	ND	ND	7.3 (4.6–13.7)	ND	7.7 (mean)
Ht (%)	ND	21 (15–30)	25 (13–33)	ND	21 (13–33)	ND
Creatinine (mg/dl)	0.9 (0.7–10.7)	1.2(0.9–5.5)	1.1 (0.7–3.1)	1.1 (0.67–5.2)	1.6 (0.7–6.6)	1.6 (mean)
BUN (mg/dl)	23.4 (2.5–154)	ND	ND	ND	ND	ND
Fever (%)	72	ND	31	36	ND	70
CNS involvement (%)	79	50	56	74	50	25
% Survival	84	81	81	87	78	81

ND: no data.
Median (minimum-maximum).
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deficiency, the other cohorts included smaller number of patients with idiopathic TTP and severe ADAMTS13:AC deficiency [7–9,11–13]. These age-related differences in clinical findings may account in part for higher short-term mortality rates observed among older patients with TTP in our cohort, as well as in the cohort reported from Canada [14].

Fourth, inhibitory autoantibodies against ADAMTS13 were identified in 97.8% of patients with ADAMTS13:AC deficient TTP. Other cohorts identify inhibitory antibodies in 44% to 94% of TTP patients with severely deficient ADAMTS13:AC levels [7–13]. These findings reflect variable sensitivity and specificity of ADAMTS13:AC inhibitor tests. In our study, ADAMTS13 inhibitor levels of 5 or more BU/ml were identified in 21.0% of TTP patients with severely deficient ADAMTS13:AC levels and inhibitor levels of 10 or more BU/ml were noted in 8.1%. These TTP patients with severely deficient ADAMTS13:AC activity levels and high titer inhibitors to ADAMTS13 might represent a subgroup of TTP patients for whom rituximab therapy might be particularly beneficial [12]. In general, the role of IgG antibody levels in ai-TTP is felt to be controversial. Some investigators report an association of higher titers with increased mortality, refractoriness, and more severe presentation [10,15], while others have not found similar results [7,16].

Our study has the limitation that follow-up ended at the time of hospital discharge, which prevented us from reporting on relapse rates and long-term survival rates. A second limitation is that TTP patients who were not severely deficient in ADAMTS13:AC levels were not included in this study. As noted by others, this is a heterogenous group of patients- many of whom have diseases other than TTP. Another limitation is that while our laboratory is a distinguished referral center for TMAs in Japan, it is not mandatory that information on all TMA patients is sent to our laboratory, and hence a number of patients with TMAs in Japan are not entered into our database. A final limitation is that cohorts in two of the five comparison studies (from Korea and Oklahoma) included a minority of individuals with TTP who did not have primary idiopathic TTP [10,13].

In summary, findings from this cohort of TTP patients in Japan with severe ADAMTS13:AC deficiency parallel those reported from TTP cohorts in Europe, the United States, Canada, the United Kingdom, and Korea in several ways, but also provide insights that have not been reported previously [7–14]. Novel findings in this cohort include females accounting for only 54.8% of incident cases, a higher median age at TTP onset of 54 years, and higher mortality rates amongst patients who were older than 60 years of age. Given the rarity of TTP in the general population, aggregation of findings from various TTP cohorts reported from Japan, Korea, France, England, Saint Louis, Oklahoma, and Canada might yield important findings that single registries would be unable to identify. A particularly important finding might be development and validation of a multivariate model predictive of mortality of persons with incident TTP characterized by severe ADAMTS13:AC deficiency.

Methods

Since 1998, our laboratory has been a nationwide referral center within Japan for TMAs, with 919 patients having been registered in this database [17]. During the first years of the study, samples from all TMA patients were evaluated by our referral center. In recent years, commercial laboratories now provide access to ADAMTS13:AC evaluation and some centers therefore do not submit samples to our group. We are not able to ascertain which centers are sending samples to commercial vendors at this time. Of these 919 patients, 186 patients were diagnosed with first onset of ai-TTP characterized by severe deficiency of ADAMTS13:AC (<5%) and no prior history of TTP. Exclusion criteria were exposure to drugs that cause TTP or TMA, organ transplantation, stem cell transplantation, immunologic disease, or ADAMTS13:AC levels 5% and more. All patients gave written informed consent to participate in this study. The study protocol was approved by the Ethics Committee of Nara Medical University Hospital.

Diagnostic criteria

The classic pentad for TTP was defined as follows (i) microangiopathic hemolytic anemia (hemoglobin $\leq$12 g/dL), Coombs test negative, undetectable serum haptoglobin (<10 mg/dL), more than 2 fragmented red cells (schistocytes) in a microscopic field with a magnification of 100, and concurrent increased serum lactate dehydrogenase (LDH) above institutional baseline, (ii) thrombocytopenia (platelet count $\leq 100\times10^9$/L), (iii) fever $\geq$37°C, (iv) CNS involvement: ranging from headache to coma, including neurological dysfunction, convulsion, clouding of consciousness, and (v) renal involvement (including abnormal urinalysis in addition to elevation of serum creatinine level). Patients were excluded if they reported a prior episode of ai-severely ADAMTS13-deficient TTP (n = 18 patients).

Blood Sampling

Before therapeutic approaches were initiated, whole blood samples (~5 ml) were phlebotomized from each patient and placed into plastic tubes containing 1/10 volume of 3.8% sodium citrate. The plasma was separated by centrifugation at 3000 g for 15 min at 4°C, kept in aliquots at −80°C until testing, and sent to our laboratory with clinical information.

Assays of plasma ADAMTS13:AC and ADAMTS13:INH

Until March 2005, ADAMTS13:AC was determined by classic von Willebrand factor multimer (VWFM) assay with a detection limit of 3% of the normal control [18,19]. Thereafter, a chromogenic ADAMTS13-act-ELISA [20] with a detection limit of 0.5% of the normal control was developed, and replaced the VWFM assay. Thus, most of the plasma samples stored at −80°C were re-evaluated with chromogenic act-ELISA, but 22 samples were unable to evaluate by the new method, because of a short of the stored sample volume. Basically, however, the results obtained by both the assays had a high correlation (r = 0.99) [20]. Thus, the results determined by VWFM alone were also included in this study. Further, to compare the results from other investigators and potentially with different assay methods for ADAMTS13:AC, we here categorized plasma levels of ADAMTS13:AC of severe (<5%), moderate (5%~<25%), mild (25%~<50%) deficiency and normal ($\geq$50%) of ADAMTS13:AC. Plasma ADAMTS13:INH titers were analyzed either by classic VWFM assay or chromogenic ADAMTS13-act-ELISA using heat-inactivated plasmas at 56°C for 30 min. Briefly, the tested samples were mixed with an equal volume of the normal plasmas and incubated at 37°C for 2 hours. After incubation, the residual ADAMTS13:AC was measured. One Bethesda unit (BU) is defined as the amount necessary to reduce ADAMTS13:AC to 50% of control levels according to the Bethesda method, which was originally developed for the measurement of factor VIII inhibitor [21]. Titers $\geq$0.5 BU/ml were classified as inhibitor-positive.

Statistical analysis

All continuous variables were reported as median values (25, 75 percentile). Comparisons between two patient groups (severe

deficiency and detectable ADAMTS13 activity) were tested for statistical significance using the Mann-Whitney U-tests or chi-square tests. Comparisons between 4 patients groups (under 20 years old, 20 to under 40 years old, 40 to under 60 years old, and over 60 years old) were calculated using the Kruskal-Wallis H tests or chi-square tests with Yates' correction for 2×4 tables. Significant differences between 4 groups (overall $p<0.05$) were further analyzed by Mann-Whitney U-tests or chi-square tests. Correlation between ADAMTS13:AC and :INH was analyzed by Sperman's correlation. A two-tailed P value less than 0.05 was considered to be significant.

Author Contributions

Conceived and designed the experiments: YF. Performed the experiments: AI YH MH YY HY. Analyzed the data: MM. Wrote the paper: MM CB YF ZQ.

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CASE REPORT

Two newborn-onset patients of Upshaw–Schulman syndrome with distinct subsequent clinical courses

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Abstract Upshaw–Schulman syndrome (USS) is caused by a congenital deficit in ADAMTS13 activity owing to genetic mutations. USS is characterized by severe neonatal jaundice with a negative Coombs test and repeated childhood episodes of thrombocytopenia reversible by fresh frozen plasma (FFP) infusions. We present two patients with USS, both of whom underwent exchange blood transfusions as newborns, although the disease subsequently developed along different clinical courses. USS-CC5 initially received a diagnosis of neonatal jaundice due to fetomaternal ABO incompatibility with an indirect positive Coombs test, which masked the diagnosis of USS. Before prophylactic FFP infusions were initiated, USS-CC5 had chronic thrombocytopenia. In contrast, thrombocytopenia developed in USS-HH4 only in response to infections and spontaneously normalized without FFP infusions. Analyses of the ADAMTS13 genes in USS-CC5 and USS-HH4 revealed compound heterozygotes of p.R398C/p.Q723K and p.Q449X/p.Q1374Sfs, respectively. Analysis of von Willebrand factor (VWF) multimers in plasma samples taken from both patients in remission showed single symmetrical multimer bands, which differ from the triplet structure of bands observed in normal samples. These data suggested that plasma VWF multimers in the patients had not been proteolytically modified. Our results indicate the presence of a previously unknown regulatory mechanism for VWF-dependent high-shear stress-induced platelet aggregation.

Keywords Upshaw–Schulman syndrome · Fetomaternal ABO incompatibility · ADAMTS13 gene analysis · Von Willebrand factor multimers

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Introduction

Upshaw–Schulman syndrome (USS) is caused by mutations in the ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs-13) gene that disrupt the activity of the encoded enzyme; the disorder is also referred to as congenital thrombotic thrombocytopenic purpura (TTP) [1–4]. Reduced ADAMTS13 activity results in increased circulating levels of unusually large von Willebrand factor multimers (UL-VWFMs), which cause microcirculatory platelet thrombi in response to high-shear stress. Although approximately 100 patients with USS have been identified in 80 families worldwide, the precise incidence of this rare disease is still unknown [5]. Kokame et al. [6] recently analyzed ADAMTS13 cDNA sequences and found rare non-synonymous ADAMTS13 gene mutations in 128 normal Japanese individuals, leading to estimates of 80–160 patients with USS among the 138 million individuals in the Japanese population.

USS typically results in severe neonatal jaundice with a negative Coombs test and a requirement for exchange blood transfusion, and repeated childhood episodes of thrombocytopenia that are reversed by infusing fresh

frozen plasma (FFP) [7]. More recently, however, two distinct clinical phenotypes have been identified in patients with USS: the major population exhibits the early-onset phenotype that includes newborn-onset disease, whereas the minor population shows the late-onset phenotype, in which overt TTP develops after adolescence [4, 8].

In this paper, we describe two young, unrelated patients with USS who both had severe neonatal jaundice that required exchange blood transfusions. The subsequent clinical courses of disease in these individuals, however, differed; one patient requires periodic prophylactic plasma infusions, whereas the other does not. Further, fetomaternal ABO incompatibility masked the diagnosis of USS in the first patient. We performed a comparative study of the two patients with USS and their family members, including ADAMTS13 genotyping, VWF multimer analysis during remission and the natural histories of disease.

Materials and methods

Patients

Clinical and laboratory data for two unrelated patients with USS (cases USS-CC5 and USS-HH4) and their family members are described in the "Results" section.

Analyses of VWF antigen and VWF multimers

Plasma VWF antigen levels were measured using sandwich enzyme-linked immunosorbent assays (ELISAs) and rabbit anti-human VWF polyclonal antiserum (DAKO, Denmark) [9]. VWF antigen levels in 1 mL of pooled normal human plasma were defined as 100 %. VWF antigen levels in the 20 healthy control subjects were 102 ± 33 % (mean ± SD) [10].

Analysis of VWF multimers was performed according to the method described by Ruggeri and Zimmerman [11], with the following modifications. Briefly, plasma samples were separated by electrophoresis on sodium dodecyl sulfate (SDS)-1.2 % agarose gels, and samples were subjected to Western blotting with anti-VWF polyclonal antibodies and luminographic detection [12]. Blots were scanned and densitometric analyses were performed using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Assays of ADAMTS13 activity and ADAMTS13 inhibitor levels

Plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor were measured using a chromogenic activity ELISA (ADAMTS13-act-ELISA; Kainos, Tokyo) [13]. ADAMTS13 activity in pooled normal plasma was defined as 100 %. The detection limit of the assay was 0.5 % of normal values. ADAMTS13 inhibitor titers are expressed as Bethesda units (BU); one unit was defined as the amount necessary to reduce ADAMTS13 activity to 50 % of control levels. A titer of <0.5 BU/mL in the assay was considered negative.

Assays of ADAMTS13 antigen

Plasma levels of ADAMTS13 antigen were determined in quantitative sandwich ELISAs using two anti-ADAMTS13 monoclonal antibodies, as previously reported [14]. ADAMTS13 antigen levels in pooled normal plasma were defined as 100 %. The detection limit of the assay was 0.1 % of normal values. Plasma ADAMTS13 antigen levels were also analyzed quantitatively and qualitatively on Western blots under reducing conditions [15]. Two milliliters of diluted or undiluted plasma samples were added to each lane and separated using SDS-5 % polyacrylamide gel electrophoresis under reducing conditions. Proteins were electrophoretically blotted onto microporous polyvinylidene difluoride membranes. Blots were probed for ADAMTS13 antigen using WH2-11-1 as primary monoclonal antibodies, and horseradish peroxidase-conjugated goat anti-mouse IgG as secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MO, USA). The epitope for WH2-11-1 antibodies resides in the fourth TSP1 domain. After incubations with Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Shelton, CT), blots were exposed to X-ray films. Densitometric analysis of ADAMTS13 antigen was performed by examining the 190-kD band using ImageJ software. The detection limit of plasma ADAMTS13 antigen using this method was 3 % of normal values.

ADAMTS13 gene analysis

All DNA analyses were performed with permission from the Ethics Committees of the hospitals at which the samples were collected and the institute where the genes were analyzed. Written informed consent was obtained from all subjects. Nucleotide sequences for all 29 exons of ADAMTS13, including intron–exon boundaries, were determined by directly sequencing polymerase chain reaction products, as previously described [16, 17]. All disease-causing ADAMTS13 mutations described in this paper were determined not to be common polymorphisms based on screening 96 individuals from the Japanese general population.

Results

Patients

Case USS-CC5

The propositus was born in 2004 in Nihonkai General Hospital, the last of three offspring of non-consanguineous parents. The delivery was natural after a gestation period of 37 weeks and 6 days and the newborn weighed 2,750 g. Eleven hours after delivery, the baby developed severe jaundice (total bilirubin 17.6 mg/dL at 12 h after delivery) and petechiae. The direct Coombs test was negative, whereas the indirect Coombs test was positive. Further, a weak anti-B antibody signal was detected in antibody dissociation experiments using the patient's red blood cells. His blood type was B-Rh(D), whereas that of his mother was O-Rh(D). Therefore, the patient was suspected of having newborn hemolytic anemia due to fetomaternal ABO incompatibility. Two exchange blood transfusions using mixed blood containing O-Rh(D) red blood cells and AB-Rh(D) FFP were performed followed by phototherapy. Subsequently, the patient's platelet count dropped to 7×10^9 platelets/L, and platelet concentrates (total of 8×10^{10} platelets on two occasions) were infused without any notable adverse reactions. The jaundice gradually improved, although his platelet count remained low ($40–60 \times 10^9$ platelets/L). The patient was discharged 7 days after birth.

At 7 months of age, the patient was infected with influenza A and showed mild anemia (hemoglobin 7.8 g/dL) and thrombocytopenia (10×10^9 platelets/L). Laboratory tests showed elevated serum levels of lactate dehydrogenase (LDH) (1,502 IU/L), low haptoglobin levels (<10 mg/dL), and negative Coombs tests. Examination of the bone marrow revealed no significant abnormality. Thus, a diagnosis of chronic idiopathic thrombocytopenic purpura was made, and high-dose intravenous IgG therapy was initiated, although no marked increase in platelet counts was noted ($20–50 \times 10^9$ platelets/L).

Since then, the patient has developed repeated episodes of thrombocytopenia, particularly together with febrile conditions. He received one more treatment with intravenous IgG and steroid, without any notable benefit. In fact, his plasma levels of LDH remained high (446–1,502 IU/L), and platelet counts were low ($20–50 \times 10^9$ platelets/L). Further, at the age of 2 years and 8 months, he suddenly developed a transient speech impediment and incomplete right hemiparesis, which spontaneously resolved within a few hours; head computed tomography scans revealed no notable abnormalities. Because of the unusual clinical history, the patient was referred to Nara Medical University at the age of 2 years and 9 months for ADAMTS13 analysis. A severe deficiency of ADAMTS13 activity (<0.5 % of normal values) and lack of ADAMTS13 inhibitor (< 0.5 BU/mL) were confirmed, suggesting a diagnosis of USS. The patient has since been receiving prophylactic FFP infusions every 2 weeks, which have produced transient increases in the platelet count (Fig. 1). Plasma ADAMTS13 activity levels in his father, mother and two older brothers were 46, 30, 40, and 23 % of normal values, respectively.

ADAMTS13 gene analysis

The patient was found to be a compound heterozygote for two mutations in the ADAMTS13 gene: p.R398C (c.1192 C > T, exon 10) and p.Q723K (c.2167 C > A, exon 18). The parents and two older brothers were heterozygous carriers of one of the two mutations. No single nucleotide polymorphisms that caused a missense mutation were found in the patient or his family members.

Plasma levels of ADAMTS13 based on antigen ELISAs were <0.1 % of normal values in the patient, and 24, 36, 23 and 36 % of normal values in the father, mother and two siblings, respectively. Further, plasma levels of ADAMTS13 antigen based on Western blotting were <3 % of normal values in the patient, and 36, 34, 38, and 38 % of normal values in the father, mother and two siblings, respectively (Fig. 2).

Case USS-HH4

In 2003, the proposita was born in a neighboring maternity clinic as the second of two offspring of non-consanguineous parents. Her delivery was assisted with a vacuum extractor after a gestation period of 40 weeks and 2 days. The newborn weighed 3,018 g. One day after birth, she was transferred to Nihonkai General Hospital because of severe jaundice (total bilirubin 23.7 mg/dL at 27 h after delivery), cyanosis, and thrombocytopenia (10×10^9 platelets/L). Direct and indirect Coombs tests were negative. Her blood type was AB-Rh(D), whereas those of her father and mother were A-Rh(D) and B-Rh(D), respectively. Thus, the etiology of the severe jaundice was unclear. After admission, she was received a total of four exchange blood transfusions using a mixed blood containing O-Rh(D) red blood cells and AB-Rh(D) FFP. She also underwent four platelet transfusions (a total of 14×10^{10} platelets). Subsequently, she developed transient renal insufficiency (blood urea nitrogen, 32.4 mg/dL; creatinine, 1.0 mg/dL). She also had patent ductus arteriosus, which was treated with surgical ligation 29 days after birth to prevent congestive heart failure. During the perioperative period, she received FFP infusions to replenish hemostatic factors, and was discharged 48 days after birth.

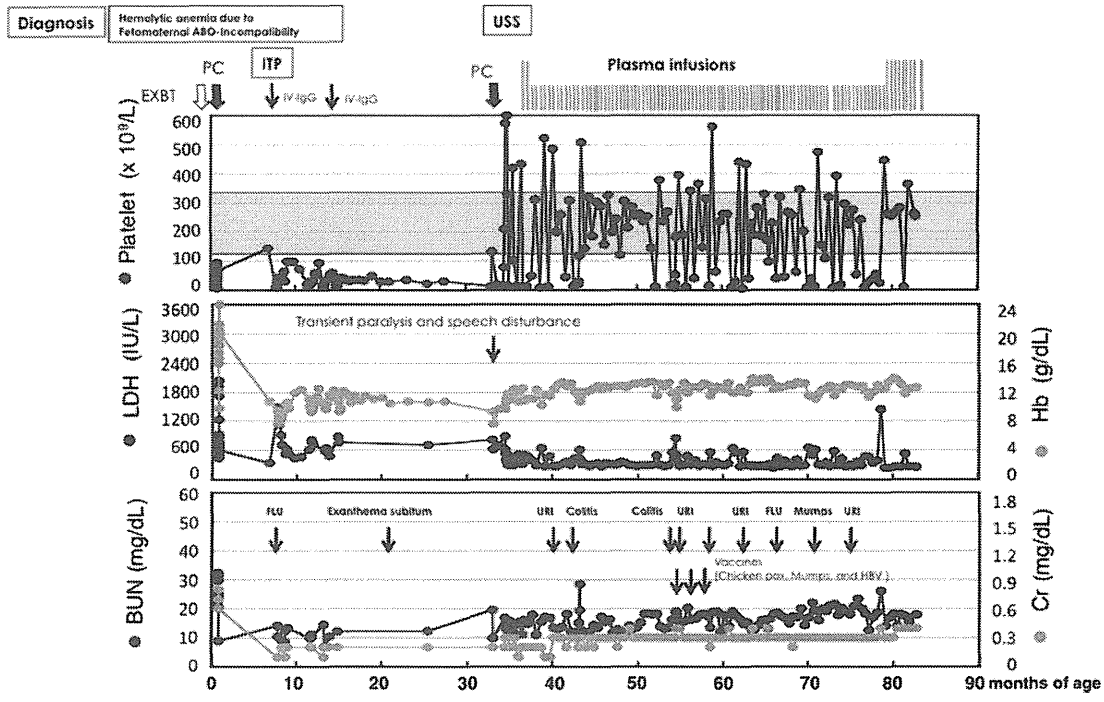

Fig. 1 Laboratory data and the clinical course of USS in patient CC5. The patient was a male born in 2004 in Nihonkai General Hospital. Soon after birth, he developed severe jaundice and petechiae. He received two exchange blood transfusions and phototherapy. Platelet concentrates were infused twice to address thrombocytopenia of unknown etiology. At 7 months of age, he received a misdiagnosis of chronic idiopathic thrombocytopenic purpura. At the age of 2 years and 9 months, he was received a diagnosis of USS caused by severely deficient ADAMTS13 activity (<0.5 % of normal values) and no ADAMTS13 inhibitors (<0.5 BU/mL). Since the USS diagnosis, the patient has received prophylactic FFP infusions every 2 weeks. Marked, yet transient, increases in platelet counts and decreases in LDH levels were observed 2–3 days after the FFP infusions. *BUN* blood urea nitrogen, *Cr* creatinine, *Hb* hemoglobin, *EXBT* exchange blood transfusion, *PC* platelet concentrate, *IV-IgG* intravenous immunoglobulin, *ITP* idiopathic thrombocytopenic purpura, *USS* Upshaw–Schulman syndrome, *FLU* influenza A infection, *URI* upper respiratory infection

At 14 months of age, she developed chicken pox with mild thrombocytopenia, and a few weeks later presented with upper respiratory infection with a fever and cough, followed by severe thrombocytopenia (17×10^9 platelets/L) and elevated serum levels of LDH (1,007 IU/L). A test for C-reactive protein was negative. Examining her bone marrow revealed hemophagocytosis in 3.8 % of the nucleated cells; subsequent fluid therapy increased her platelet count to 144×10^9 platelets/L, resulting in a preliminary diagnosis of viral infection-associated hemophagocytic syndrome. One month later, however, she developed mild anemia (hemoglobin, 9.7 g/dL) and moderate thrombocytopenia (75×10^9 platelets/L), which was not specifically treated. She then developed several episodes of petechiae with fever due to upper respiratory infections. Of note, she was infected with influenza A at 2 years and 2 months old, which induced severe thrombocytopenia that gradually resolved after administration of the anti-influenza drug oseltamivir. After she became 3 years old, the incidence of petechiae decreased together with the frequency of febrile episodes. Because of the recurrent episodes of purpura, the patient was referred to Nara Medical University for ADAMTS13 analysis in 2008. She received a diagnosis of USS based on severe deficiency of ADAMTS13 activity (<0.5 % of normal values) and a lack of ADAMTS13 inhibitor. Plasma ADAMTS13 activity levels in her father, mother and older brother were 50, 44 and 38 % of normal values, respectively.

Although she had a history of severe neonatal jaundice followed by an exchange blood transfusion, the patient did not receive FFP infusions outside of the newborn period owing to the mild clinical signs and symptoms of her disease. She is now 8 years old and has not developed any major complications, such as renal insufficiency or neurologic abnormalities (Fig. 3).

ADAMTS13 gene analysis

Patient USS-HH4 was found to be a compound heterozygote for ADAMTS13 gene mutations: p.Q449X (c.1345 C > T, exon 12) was inherited from her father and p.Q1374Sfs (c.4119del, exon 29), which resulted in premature stop codon at amino-acid residue 1395, was inherited from her mother. Her parents were heterozygous