Severe Hemolysis and Pulmonary Hypertension in a Neonate With Upshaw–Schulman Syndrome

Nobuyuki Tsujii, MD, a, b Isao Shiraishi, MD, PhD, b Koichi Kokame, PhD, c Midori Shima, MD, PhD, a Yoshihiro Fujimura, MD, PhD, d Yukihiro Takahashi, MD, PhD, e Masanori Matsumoto, MD, PhD d

Departments of aPediatrics, and dBlood Transfusion Medicine, and eDivision of Neonatal Intensive Care, Nara Medical University, Nara, Japan; and Departments of bPediatric Cardiology, and cMolecular Pathogenesis, National Cerebral and Cardiovascular Center, Osaka, Japan

Dr Tsujii provided patient information and drafted the initial manuscript; Dr Shiraishi contributed to gathering of patient data; Dr Kokame performed the ADAMTS13 gene analysis; Dr Shima contributed to gathering of patient data; Dr Fujimura conceptualized the study; Dr Takahashi contributed to gathering of patient data; Dr Matsumoto designed the study and prepared the manuscript; and all authors approved the final manuscript as submitted.

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Address correspondence to Masanori Matsumoto, MD, PhD, Department of Blood Transfusion Medicine, Nara Medical University, 840 Shijyo-cho, Kashihara, Nara 634-8522, Japan. E-mail: mmatsumo@naramed-u.ac.jp

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Upshaw–Schulman syndrome (USS) is a rare hereditary deficiency in the activity of von Willebrand factor (VWF)-cleaving protease, termed ADAMTS13. USS is a synonym for congenital thrombotic thrombocytopenic purpura (TTP). The characteristics of neonatal onset of USS are severe neonatal jaundice that requires exchange blood transfusion and repeated episodes of thrombocytopenia. TTP is diagnosed by the deficiency of ADAMTS13 activity (<10% of normal) due to mutations in the ADAMTS13 gene or autoantibodies against ADAMTS13 (inhibitors).

ADAMTS13 is a metalloprotease that specifically cleaves unusually large VWF multimers (UL-VWFMs), the most active form of VWF. UL-VWFMs are secreted from endothelial cells and are cleaved into smaller fragments just after secretion by ADAMTS13 under the high shear conditions in the microvasculature. When ADAMTS13 activity is markedly decreased, excessive amounts of UL-VWFMs result in platelet aggregation by binding platelets. Platelet thrombi induce ischemia in many systemic organs, which in turn causes TTP. However, pulmonary disease due to platelet thrombi has been reported rarely.

abstract

Pulmonary involvement is extremely rare in thrombotic thrombocytopenic purpura. In this report, we present a girl patient with congenital thrombotic thrombocytopenic purpura, known as Upshaw–Schulman syndrome (USS), complicated with severe hemolysis and pulmonary hypertension (PH). The assay results of a disintegrin-like and metalloprotease with thrombospondin type 1 motifs 13 (ADAMTS13) activity measured by FRETS-VWF73 and ADAMTS13-act-ELISA were different. Hyperbilirubinemia (total bilirubin, 25.3 mg/dL) interfered strongly with the FRETS-VWF73 assay. Plasma levels of ADAMTS13 activity by act-ELISA were <0.5% of normal. The diagnosis of USS was confirmed by ADAMTS13 gene analysis, which showed compound heterozygous mutations of p.G139Vfs*17 and p.I673F. The p.G139Vfs*17 mutation was previously unreported, and its effect in splicing was confirmed by reverse transcription polymerase chain reaction. The patient received oxygen therapy for PH and exchange blood transfusion for severe hemolysis. The PH resolved without specific treatment. Based on these findings, the PH may have been caused by free hemoglobin that scavenged nitrogen oxide or platelet thrombi in the lungs caused by ADAMTS13 deficiency. Thus, severe PH can occur in neonatal patients with USS, and severe hemolysis might result in overestimation of ADAMTS13 activity. Both possibilities are important for the diagnosis and management of USS.
We report a patient with USS of neonatal onset who developed concomitant pulmonary hypertension (PH). We had difficulty measuring plasma ADAMTS13 activity by fluorescence resonance energy transfer substrate (FRETS)-VWF73 assay in this patient because of the interference of hyperbilirubinemia. Her diagnosis was confirmed by compound heterozygous ADAMTS13 mutations. One of these mutations was previously unreported, and the effect on splicing was analyzed by reverse transcription polymerase chain reaction (RT-PCR).

**METHODS**

Plasma ADAMTS13 activity was measured by both FRETS-VWF73 assay (Fluorescence-Quenching Substrate for ADAMTS13)\[7\] and chromogenic ADAMTS13 activity enzyme-linked immunosorbent assay (ELISA; Kainos Laboratories, Tokyo, Japan).\[8\] FRETS-VWF73 (Peptides International, Ibaraki, Osaka, Japan) assay measures the absorbance change in the mixture of the standard or test plasma and the substrate solution in a 96-well plate with excitation wavelengths of 340 nm and emission wavelengths of 450 nm; the ADAMTS13 activity is calculated from the reaction rate (slope) by linear regression of fluorescence. In contrast, the chromogenic act-ELISA assay is a sandwich ELISA method that uses a VWF73 substrate (Glutathione S-transferase [GST] - VWF73- Histidine [His]) coupled to anti-GST monoclonal antibodies on a solid-phase microplate and horseradish peroxidase (HRP)-conjugated anti-N10 monoclonal antibodies, which recognize the C-terminal edge of the VWF A2 domain cleaved specifically by ADAMTS13. One Bethesda unit (BU) of inhibitor was defined as the amount of inhibitor that reduced ADAMTS13 activity to 50% of control.\[3\] The study protocol was approved by the ethics committee of Nara Medical University, and written informed consent was obtained from the infant's parents.

**CASE PRESENTATION**

A baby girl without a family history of thrombotic and hemorrhagic diseases was born at 39 gestational weeks with a body weight of 3470 g. Six hours after birth, she developed jaundice and hypoxemia (oxygen saturation using pulse oximetry was 89%). She was diagnosed with suspected aortic coarctation and referred to the National Cerebral and Cardiovascular Center on day 1. Ecchymosis on the back and legs, gross hematuria, and severe jaundice were observed. Transthoracic echocardiography (TTE) ruled out structural heart disease but revealed bowing of the intraventricular septum into the left ventricle, slight tricuspid regurgitation with a pressure gradient of 49 mm Hg, and patent ductus arteriosus with right to left shunt in early systolic phase, indicating severe PH (Fig 1). Patent foramen ovale with left to right shunt and preserved both ventricle wall motion of both ventricle were also revealed (ejection fraction of left ventricle was 88%).

Laboratory findings on admission revealed severe thrombocytopenia (3.0 [normal range, 150–350] × 10^9/L), elevated D-dimer (12.8 [normal range, <1.0] μg/mL), and indirect hyperbilirubinemia (unconjugated bilirubin 24.9 [normal range, 3.4–5.0] μg/dL), with schistocytes on blood film (Table 1). The findings of thrombocytopenia and severe jaundice in a newborn led to the suspicion of USS. To confirm this diagnosis, plasma ADAMTS13 activity was first measured by FRETS-VWF73 assay. Although ADAMTS13 activity was around 10% of normal, we were concerned about the possibility that hyperbilirubinemia may have interfered with fluorescence and thus led to spurious results. Therefore, chromogenic ADAMTS13 act-ELISA was also performed. This assay, which is unaffected by bilirubin levels up to 50 mg/dL,\[10\] showed that ADAMTS13 activity was <0.5% of normal, and the level of its inhibitor was 0.9 BU/mL, possibly due to hyperbilirubinemia. Therefore, the infant was strongly suspected of having USS. Later, we confirmed the ADAMTS13 activity of the patient by classic VWF multimer assay using full-length VWF,\[11,12\] which showed <3% of normal.

The patient received oxygen therapy for PH and exchange blood transfusion for severe hemolysis and hyperbilirubinemia. Exchange blood transfusion with red blood cells, fresh frozen plasma (FFP), and platelet concentrate was performed to prevent critical hemorrhage. She simultaneously received phototherapy and intravenous infusion of immunoglobulin (0.5 g/kg) and haptoglobin (250 and 100 U/kg). After the exchange blood transfusions were complete, we continued to infuse FFP for an additional 3 days. Platelet counts increased, schistocytes were not detected on blood film, and D-dimer was reduced to 6.3 μg per mL on day 4. On day 5, she had a tonic convolution without computed tomographic findings of thrombosis or ischemia in the brain. No additional convulsions occurred. Based on the TTE findings, PH resolved on day 7 despite the lack of specific treatment. The patient has received FFP infusion every 3 weeks for TTP prophylaxis.

**ADAMTS13 GENE ANALYSES**

The patient’s plasma ADAMTS13 activity was measured before FFP infusion on 11 separate occasions by ADAMTS13 act-ELISA. The values were always <0.5% of normal and ADAMTS13 inhibitors were detected only on day 1. Plasma ADAMTS13 activity in the patient’s father and
FIGURE 1
Clinical course and echocardiography findings. The upper panel shows TTE results. On days 1 and 3, the intraventricular septum (IVS) bows into the left ventricle (LV). This finding is not seen on days 5 and 7. IVIg, intravenous immunoglobulin; RV, right ventricle.
mother was 41.5% and 42.6%, respectively. The normal level of ADAMTS13 activity determined by ADAMTS13 act-ELISA from 55 healthy individuals is 99.1% ± 21.5 (mean ± SD). To confirm the diagnosis of USS, direct sequencing of all exons and flanking intronic regions of ADAMTS13 was performed in the patient and her parents using methods reported previously. We found 3 mutations, namely, c.415-10G>A, c.824+13C>T, and c.2017A>T (p.I673F). Of these, c.824+13C>T was reported as a variant (rs149586801) with a global minor allele (T) frequency of 0.023 (1000 Genomes data). Consequently, the patient was a compound heterozygote for ADAMTS13 mutations c.415-10G>A, inherited from her mother, and c.2017A>T (p.I673F), inherited from her father. The p.I673F mutation was reported previously.

To determine the effect of c.415-10G>A on splicing, we analyzed RNA prepared from the patient’s blood (Fig 2). RT-PCR using 2 primers, 5′-AACCTCAACATCGGGGCAG-3′ (exons 3 and 4) and 5′-CGTCCTCAGGGTTGATGTC-3′ (exon 5), produced a single band in a control subject and double bands in the patient (Fig 2A). Sequencing the products after cloning revealed that the lower band (178 bp) was normal ADAMTS13 cDNA and the upper band (186 bp) contained an 8-bp insertion (Fig 2B), which was probably caused by a new splice acceptor site produced by the c.415-10G>A mutation. The frame shift caused by the 8-nucleotide insertion replaced Gly139 with Val, with a stop codon after 15 amino acid residues (p.G139Vfs*17).

**DISCUSSION**

In this report, we present a neonatal patient with USS who suffered from severe hemolysis and PH. To the best of our knowledge, PH complicated by USS has not been previously reported. Pulmonary involvement in patients with TTP is extremely rare. In this case, the cause of the PH may have been severe hemolysis, which led to scavenging of nitric oxide (NO) and pulmonary thrombosis. Hemolysis is associated with PH, especially in patients with sickle cell anemia. When severe hemolysis occurs intravascularly, free hemoglobin is released into plasma and scavenges NO. NO reacts at least 1000 times more rapidly with cell-free hemoglobin than hemoglobin within erythrocytes. NO causes pulmonary dilatation and inhibition of endothelial cell damage. Therefore, decreased NO caused by severe hemolysis leads to PH. High levels of hemoglobin F are reported to be associated with a lower hemolytic rate of erythrocytes, and thus hyperbilirubinemia caused by hemolysis in the neonatal period tends to be milder than that in adulthood because of elevated hemoglobin F levels. In this case, however, severe hyperbilirubinemia was detected even in the neonatal period. This might indicate that overly severe hemolysis had developed in this patient.

Pulmonary thrombosis has rarely been reported in patients with TTP. There are several possible

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**TABLE 1 Laboratory Findings on Admission**

<table>
<thead>
<tr>
<th>Hematologic Examination</th>
<th>Normal</th>
</tr>
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<tbody>
<tr>
<td>White blood cell</td>
<td>27 × 10^9/μl (4.0–9.0)</td>
</tr>
<tr>
<td>Red blood cell</td>
<td>4290 × 10^9/μl (5800–5100)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>13.9 g/dL (12.0–16.5)</td>
</tr>
<tr>
<td>Reticulocyte</td>
<td>38‰ (5–20)</td>
</tr>
<tr>
<td>Platelets</td>
<td>3 × 10^9/L (150–350)</td>
</tr>
<tr>
<td>Schistocytes on blood film</td>
<td>+++ —</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hemostatic test</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT:INR</td>
<td>1.38 (0.90–1.14)</td>
</tr>
<tr>
<td>A:PTT</td>
<td>45 s (24–40)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>248 mg/dL (200–400)</td>
</tr>
<tr>
<td>Antithrombin</td>
<td>54% (80–130)</td>
</tr>
<tr>
<td>Fibrin degradation products</td>
<td>17 μg/mL (&lt;5)</td>
</tr>
<tr>
<td>D-dimer</td>
<td>12.8 μg/mL (&lt;1.0)</td>
</tr>
<tr>
<td>VWF antigen</td>
<td>115% (55–190)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Chemistry</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>5.1 g/dL (6.7–8.3)</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>25.3 mg/dL (4.0–5.0)</td>
</tr>
<tr>
<td>Conjugated bilirubin</td>
<td>0.4 mg/dL (0.0–0.4)</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>207 IU/L (15–33)</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>14 IU/L (6–27)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>3144 IU/L (119–229)</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>28 mg/dL (8.0–22.0)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1.18 mg/dL (0.4–0.7)</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>&lt;10 mg/dL (17–169)</td>
</tr>
<tr>
<td>Brain natriuretic peptide</td>
<td>745.7 pg/mL (&lt;18.4)</td>
</tr>
</tbody>
</table>

| ADAMTS13 Activity (FRETS) | 10% (50.0–150.0) |
| ADAMTS13 Activity (ELISA) | <0.5% (50.0–150.0) |
| ADAMTS13 Activity (VWF multimer method) | <3% (50.0–150.0) |
| Inhibitor | 0.8 BU/mL (<0.5) |

<table>
<thead>
<tr>
<th>Uricanalysis</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific gravity</td>
<td>1.01 (1.005–1.0025)</td>
</tr>
<tr>
<td>Protein</td>
<td>2+ —</td>
</tr>
<tr>
<td>Sugar</td>
<td>— —</td>
</tr>
<tr>
<td>Ketones</td>
<td>— —</td>
</tr>
<tr>
<td>Occult Blood</td>
<td>3+ —</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sediment</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell</td>
<td>50–99 /HPF —</td>
</tr>
<tr>
<td>White blood cell</td>
<td>30–49 /HPF —</td>
</tr>
<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>79.8 U/L 1-4.2</td>
</tr>
<tr>
<td>β2-microglobulin</td>
<td>37,600 μg/L 16-518</td>
</tr>
</tbody>
</table>
explanations for this. First, pulmonary vessels may be inherently resistant both to the development of platelet microvascular thrombi and to the occurrence of endothelial injury. Second, the low shear stress of pulmonary circulation may limit the formation of VWF-mediated thrombi. Third, the systemic deficiency of ADAMTS13 has no impact on the lungs. As previously reported, ADAMTS13 is not expressed in pulmonary tissues. In this case, elevated D-dimer on admission might indicate pulmonary thrombosis. Decreased NO and thrombosis formation in the lungs induced by ADAMTS13 deficiency might have contributed to PH. In addition, pulmonary thrombosis and decreased NO induced PH, which resolved spontaneously despite the lack of specific treatment. Therefore, PH might be a common symptom in cases of neonatal USS with severe jaundice and it should be closely considered in such cases.

We found a difference in plasma ADAMTS13 activity measured by FRETs-VWF73 assay and ADAMTS13-act-ELISA. Hyperbilirubinemia interferes with fluorescence evolution in the FRETs-VWF73 assay by acting as a quencher at the emission wavelength of 450 nm, and bilirubin concentrations >100 μmol/L (5.85 mg/dL) alter ADAMTS13 activity data. In this patient, total bilirubin was extremely high at 25.3 mg/dL (432 μmol/L). Therefore, it was difficult to correctly calculate the reaction rate using the linear regression of fluorescence in the FRETs-VWF73 assay, because this calculation overestimated the ADAMTS13 activity rather than underestimated it as in a previous report. In addition, hyperbilirubinemia directly inhibits ADAMTS13 activity. Therefore, at the patient’s admission, we detected low titer of inhibitors against ADAMTS13 (0.9 BU/mL). Although FRETs-VWF73 is a rapid and useful assay for ADAMTS13 activity, we should avoid it when serum bilirubin levels are >6 mg/dL.

The diagnosis of USS was confirmed by ADAMTS13 gene analysis. The patient had compound heterozygous mutations of p.G139Vfs*17 and p.I673F. Plasma ADAMTS13 antigen in this patient was found to be completely absent in plasma by both ELISA and Western blotting (data not shown). Therefore, the ADAMTS13 mutant involving p.G139Vfs*17 and p.I673F might have disturbed secretory mechanisms in vivo.

In conclusion, in this report, we present a newborn with USS who was diagnosed by ADAMTS13 gene analysis. She had severe hemolysis that resulted in both increased free hemoglobin and hyperbilirubinemia. The former might have been the cause of the patient’s PH, and hyperbilirubinemia interfered with the measurement of ADAMTS13 activity. PH might not be a rare phenomenon in newborns with USS and severe hemolysis.

**ACKNOWLEDGMENTS**

We thank Ms Ayami Isonishi and Dr Kazuya Sakai for excellent technical assistance and Drs Yousuke Hayama, Takashi Nakagawa, and Masahiro Takeyama for gathering patient data and samples.
ABBREVIATIONS
ADAMTS13: a disintegrin-like and metalloprotease with thrombospondin type 1 motifs
13
BU: Bethesda unit
ELISA: enzyme-linked immunosorbent assay
FFP: fresh frozen plasma
FRETS: fluorescence resonance energy transfer substrate
NO: nitric oxide
PH: pulmonary hypertension
RT-PCR: reverse transcription-polymerase chain reaction
TTE: transthoracic echocardiography
TTP: thrombotic thrombocytopenic purpura
UL-VWF: unusually large VWF
USS: Upshaw-Schulman syndrome
VWF: von Willebrand factor

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