Abnormalities in clot stability and effect of clot stabilizing intervention

Thromboelastometry complements thrombin generation measurements by being able to provide information about clot firmness and clot stability. Clot stability and resistance toward facilitated fibrinolysis can be investigated in assays containing TF + tissue plasminogen activator. Adopting such assays, thromboelastometry studies have shown effect of tranexamic acid [21] and also recently factor XIII supplementation [22].

Correlation of thromboelastography with clinical phenotype

A number of studies have demonstrated considerable heterogeneity in the baseline whole blood coagulation patterns amongst patients with verified factor VIII levels < 1% [23]. Furthermore, data have illustrated that patients diagnosed with severe haemophilia A (fVIII:C < 1%) but having unusually good whole blood clotting profiles are associated with a less severe bleeding phenotype [24].

Prediction of response to by-passing agents

The low tissue factor assay has also been used to illustrate different response patterns to various levels of coagulation factor VIII concentrate. In addition, both *in vitro* and *in vivo* studies have demonstrated the ability of thromboelastography to predict the clinical response to bypassing agents in patients with inhibitors [25–27]. A small clinical study has shown that thromboelastography may be used to individualize therapy and provide more judicious use of bypassing agents as well as more convenient treatment regimens [28]. Recently, thromboelastometry has been utilised to correct the haemostatic performance of recombinant factor VIIa during surgery by showing need for fresh platelet concentrate to secure effect of recombinant factor VIIa [29].

Future perspectives

Ongoing scientific activities aim to further standardize the use of thrombin generation and thromboelastometry for use in haemophilia. Important future questions will include source and concentration of tissue factor for the global assays.

A series of additional by-passing agents are in development [30], thus further emphasizing the need for global assay to monitoring and provide theranostic guidance.

Disclosures

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Clot waveform analysis

Clot waveform analysis (CWA) is a convenient method for assessing global clotting function. It is based on the continuous monitoring of light transmittance or absorbance during routine coagulation tests such as the activated thromboplastin time (aPTT) and the prothrombin time (PT). Numerous automated instruments are capable of performing CWA. Principally, CWA can be conveniently performed simultaneously with routine coagulation tests [1]. Changes in light transmittance are determined by continuous measurements and are designated the clot waveform (CW). This complete clotting process recorded in the CWA is categorized into three parts, e.g. the pre-coagulation phase, the coagulation phase and the post-coagulation phase. After the onset of coagulation, light transmittance is decreased in association with the formation of fibrin and is defined by a slope in the waveform. The advantages of utilizing CWA are provided by the quantitative assessment of various parameters derived by mathematically processing the waveform data. Early reports suggested, however, that |min1| and |min2| are promising parameters for quantitative evaluation of clotting function [2]. Observations of CWA patterns during routine aPTT and PT assays can provide supportive and novel data in a variety of coagulation disorders and during monitoring of anti-coagulant therapy such as heparin. Characteristic CW patterns are observed in specific coagulation abnormalities compared with normal reference plasma, and two components, the duration of pre-coagulation phase and the steepness of the slope of the coagulation phase, appear to be especially informative. A further advantage offered by the application of CWA is the possibility of assessing fibrin deficiency and fibrinolytic activity. Furthermore, modification to a "biphasic" pattern is a useful tool for diagnosis of sepsis and disseminated intravascular coagulation (DIC) [3]. CWA could discriminate between different levels of fVIII:C in this critical category of severe HA defined as having <1.0 IU dl^{-1} fVIII:C by conventional assays [2]. Furthermore, the CWA parameter, |min2|, appeared to be more directly correlated with both the degree of abnormality of the CW and the fVIII:C level [4]. Similarly, in experiments in 36 patients with severe HA, significant correlations between |min2| and fVIII:C were confirmed, and the parameters correlated well with those of thrombin generation [4,5]. It is evident that since the distinction between severe and non-severe haemophilia cannot be determined precisely by the level of fVIII or fIX activity alone, the influence of other plasma components should be considered. It may be especially important that CWA can clearly discriminate between severe and non-severe groups. Defective clotting function in haemophilia can be assessed using CWA, and this method may be applicable to monitor the haemostatic and prophylactic effects of regular infusions of fVIII concentrate during ITI therapy in patients with inhibitor. Our previous results suggested that fVIII infusions may be continued with clinical benefit in some haemophilia patients with high responding inhibitors in whom the haemostatic response may be monitored effectively using CWA [6]. Lastly, CWA is also useful for assessment of clotting function in acquired haemophilia A, since this refractory and severe bleeding disorder cannot be estimated by the level of fVIII activity [7]. Thus, CWA has greater versatility and considerable potential for the evaluation of overall clotting function in various disorders of haemostasis. Internationally recognized standardization of methods and test parameters are required, however, for optimization of the technique.

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Diagnostic usefulness of adenosine triphosphate release assays and aggregation tests with native or platelet count adjusted platelet rich plasma

Platelet function disorders are quite prevalent among individuals with bleeding problems [1–5]. At present, aggregation and dense granule release assays are the commonly performed, and the most useful tests to diagnose platelet function disorders [1–4,6,7]. Laboratories need to consider recent evidence on aggregation and dense granule release tests for platelet disorders [1–5,8], and the guideline recommendations on these assays [9–12] to optimize their diagnostic evaluation of platelet function disorders.

Light transmittance aggregometry (LTA) is considered the "gold standard" of platelet function tests, despite its lack of standardization [13,14]. The usefulness of LTA, for diagnosing impaired platelet function among individuals referred for bleeding disorder assessments, has been estimated in recent prospective studies [1,3]. A merit of these studies is that they tested LTA in accordance with guidelines [9,10], using validated reference intervals (RI) for maximal aggregation (MA) [15]. When LTA MA is abnormal with two or more panel agonists, there is a high likelihood (estimated as OR, odds ratio) of impaired function from a bleeding disorder (OR: ≥23), and an inherited secretion defect (OR: ≥91) which is the most common type of platelet function disorder [1,3]. In comparison, the bleeding time is much less useful (OR for bleeding disorders: 3.5) [1]. Most LTA abnormalities with single agonists are false positive results, not predictive of bleeding problems [1,3]. In general, LTA shows good reproducibility and less variability than dense granule release endpoints [2–4]. Receiver operator curves (ROC), which evaluate sensitivity and specificity, indicate LTA has high specificity and moderate sensitivity for inherited platelet disorders [1,3]. Abnormal findings can also reflect acquired disorders [1,3]. LTA agonists that are sensitive to common inherited platelet function defects include commonly tested agonists (i.e. Horm collagen, tested at 1.25 $\mu g\ mL^{-1}$; epinephrine; and arachidonic acid) and thromboxane analogue U46619 [1], which is used less frequently [7,14,16].

ontroversies have emerged about whether LTA should be performed using native platelet rich plasma (PRP) or PRP adjusted to a standardized platelet count as native samples show more aggregation with weak agonists [3,17–20] A recent prospective study was the first to rigorously compare these samples types for bleeding disorder diagnosis, using non-inferiority analysis of the areas under ROC for MA data, with predefined ROC area differences (<0.15 to define non-inferiority; >0 to define superiority) to evaluate detection of bleeding disorders and inherited platelet secretion defects [3]. Native and adjusted PRP show small differences in their mean MA responses to most agonists (ranges, controls: −3.3 to 5.8; patients referred for bleeding disorder assessments: −3.0 to 13.7), with native samples showing more variability with ristocetin [3]. For detecting reduced MA from bleeding disorders (with two or more agonists), native PRP were non-inferior, whereas adjusted PRP were superior, despite their wider RI with weak agonists [3]. While this study validates using either native or adjusted PRP for LTA assessments of bleeding disorders, adjusted PRP were superior to native PRP for detecting impaired LTA from bleeding disorders [3]. Furthermore, native PRP (which show more variable responses to ristocetin) have not been validated for ristocetin induced platelet aggregation assessments of von Willebrand disease [3].

North American guidelines recommend that laboratories consider a single abnormal agonist response by LTA as a potential false positive findings (except with collagen and ristocetin) as such abnormalities are not predictive of platelet function disorders [1,3,10]. On the other hand, evidence to date indicates that LTA abnormalities with multiple agonists are strongly associated with bleeding disorders (OR ≥23) and inherited platelet secretion defects (OR ≥91), which are the most common type of platelet function disorder [1,3]. Studies on the reproducibility of LTA indicate that most results (be they normal or abnormal) are confirmed on repeat testing [4]. Nonetheless, it is considered good practice to confirm abnormalities on another sample to exclude preexamination or analytical artifacts [10]. Abnormalities with multiple agonists should be considered suspicious of a platelet function disorder [10].

Like LTA, assays of dense granule adenosine triphosphate (ATP) release using Chronolume® (Chronolog Corporation, Haverston, PA, USA), a commercial luciferin–luciferase reagent containing magnesium, are helpful to detect impaired platelet function due to a bleeding disorder (OR: 17; diagnosis based on clinical opinion, not laboratory tests) or an inherited platelet disorder (OR: 128). ROC analyses indicate that like LTA, ATP release has high specificity and moderate sensitivity for inherited platelet disorders [2], with most function defects detected by the combination of: 6 μM epinephrine, 5.0 $\mu g\ mL^{-1}$ Horm collagen, and 1 μM thromboxane analogue U46619 [2]. ATP release abnormalities are predictive of platelet disorders, regardless of LTA findings (respective OR: if LTA abnormal: 261; if LTA normal: 105) [2]. However, the predictive power could been overestimated for subjects with normal LTA findings as ATP release was considered in the definition of platelet disorders [2]. Because ATP release findings show significant variability [2], abnormalities in platelet function should be confirmed on another sample. Several experts recommend performing aggregation and ATP release as separate tests because Chronolume® potentiates sub-maximal aggregation and falsely normalizes the aggregation findings for some platelet disorders (e.g. Quebec platelet disorder)

[5,21]. Furthermore, the agonists, and agonist concentrations, that are useful for LTA and ATP release differ [5]. There have not been any reported prospective studies on the diagnostic usefulness of whole blood ATP release, and ATP release assessed with native PRP or low platelet count samples. Laboratories should be aware that the sample platelet count influences how much platelet dense granule ATP is available for release.

To optimize platelet function testing, laboratories should consider the recent evidence, guidelines, and strategies that help detect common platelet function defects [1–5,8–12,22] including the use of properly determined RI (based on adequate numbers of control tests) and quality controls [14,16,23,24]. An improved diagnosis of platelet function disorders could limit the risk of false positive or negative findings worldwide.

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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

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

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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.

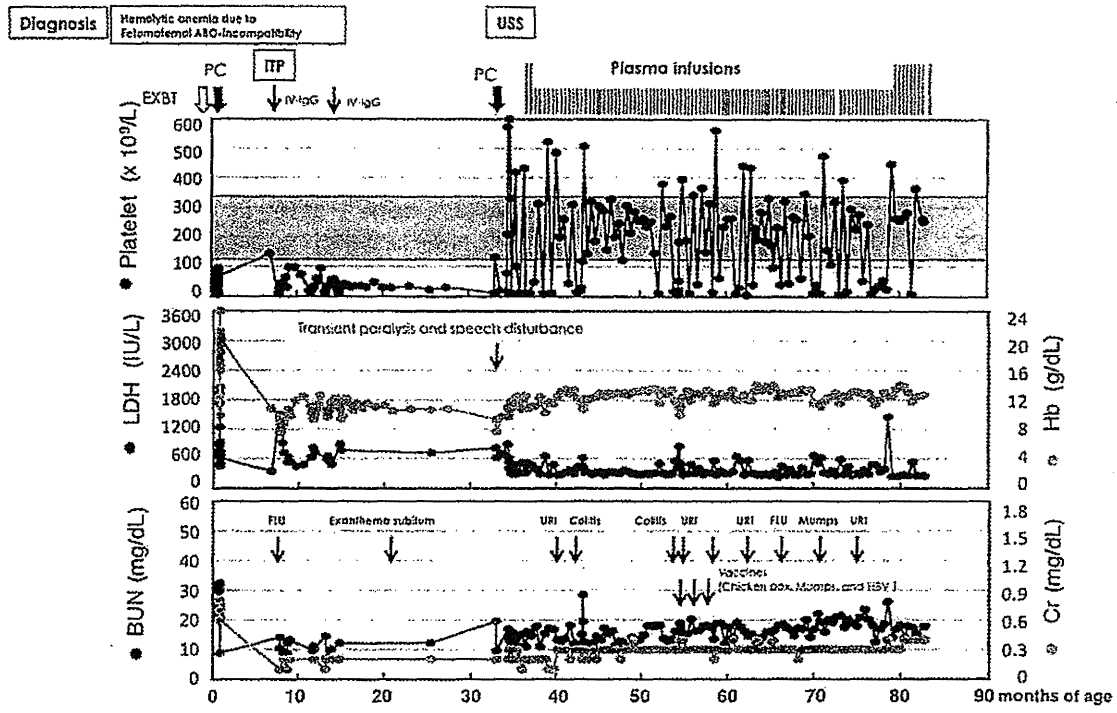


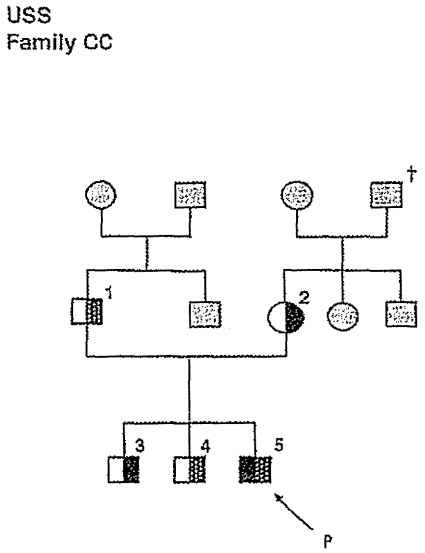
Fig. 1 Laboratory data and the clinical course of USS in patient CC5. The patient was a male born in 2004 in Nihonkai General Hospital. Soon after birth, he developed severe jaundice and petechiae. He received two exchange blood transfusions and phototherapy. Platelet concentrates were infused twice 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



	ADAMTS13 activity (%)	ADAMTS13 antigen (%)		ADAMTS13 gene	
	ELISA	ELISA	WB	p.Arg398	p.Gln723
CC-1	46	24	36	R/R	Q/K
CC-2	30	36	34	R/C	Q/Q
CC-3	40	23	38	R/C	Q/Q
CC-4	23	36	38	R/R	Q/K
CC-5	<0.5	<0.1	<3	R/C	Q/K

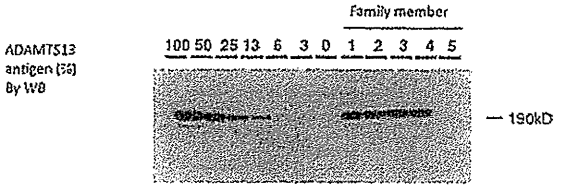


Fig. 2 Pedigree and ADAMTS13 analysis of USS-CC5 and his family. The propositus (denoted as P) USS-CC5 is the third of three offspring of non-consanguineous parents. His parents and two brothers are asymptomatic and in good health. *Squares* and *circles* indicate males and females, respectively and *shaded symbols* show individuals who were not examined. The *cross* denotes a deceased individual. *Half-black symbols* show asymptomatic carriers. ADAMTS13 activities were determined using activity ELISAs and ADAMTS13 levels were measured using antigen ELISAs and Western blotting. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted as *one-letter amino-acid abbreviations* (*right upper panel*). Western blot analyses of plasma ADAMTS13 antigen in the patient's family members are shown in the *right lower panel*

carriers of one of the two mutations. No single nucleotide polymorphisms causing missense mutations were identified in this patient or her family members.

Plasma levels of ADAMTS13 antigen based on antigen ELISAs were less than 0.1 % of normal values in the patient, whereas her father, mother and older brother showed antigen levels that were 48, 45 and 54 % of normal values, respectively. Further, plasma levels of ADAMTS13 antigen on Western blots were shown to be <3 % of normal values in the patient, and 47, 45 and 54 of normal values in her father, mother and older brother, respectively. Thus, the p.Q449X/p.Q1374Sfs mutations may have resulted in proteins that were not secreted into plasma (Fig. 4).

VWF multimer analysis of the patients with USS during remission

Despite the common features in the two USS cases, including a lack of plasma ADAMTS13 activity and severe jaundice as newborns, the subsequent clinical courses of disease markedly differed. To examine potential proteolytic mechanisms other than ADAMTS13, we performed VWF multimer analysis using plasma samples obtained when the patients were in remission. VWF multimer bands from the patients' plasma samples were each represented by a single symmetrical band, which differed from the triplet structure of bands observed with normal plasma (Fig. 5). Moreover, a predominance of high-molecular weight VWF multimers was noted in the plasma samples from both patients, suggesting that their plasma VWF multimers had not been subjected to any alternative proteolytic modifications.

Discussion

Analysis of the natural history of USS in 43 Japanese patients found that 42 % (18/43) had an episode of severe jaundice as newborns that required exchange blood transfusion, suggesting that jaundice is the earliest clinical sign of bouts of TTP in patients with USS [4]. Although the underlying mechanism has not been fully elucidated, hypoxia can induce the release of UL-VWFMs from vascular endothelial cells by upregulating the production of such inflammatory cytokines as interleukin-6, interleukin-8 and tumor necrotizing factor α [18, 19]. Moreover, newborns can be subjected to hypoxic conditions during prolonged labor, which may induce the release of UL-VWFMs from vascular endothelial cells and cause TTP bouts.

Newborns with USS often show severe jaundice with a negative Coombs test that requires exchange blood transfusion as well as repeated childhood episodes of thrombocytopenia that resolve in response to FFP infusions. Thus, patients with newborn-onset USS have been categorized as having the early-onset phenotype, and are treated throughout their lives with occasional or periodic plasma infusions

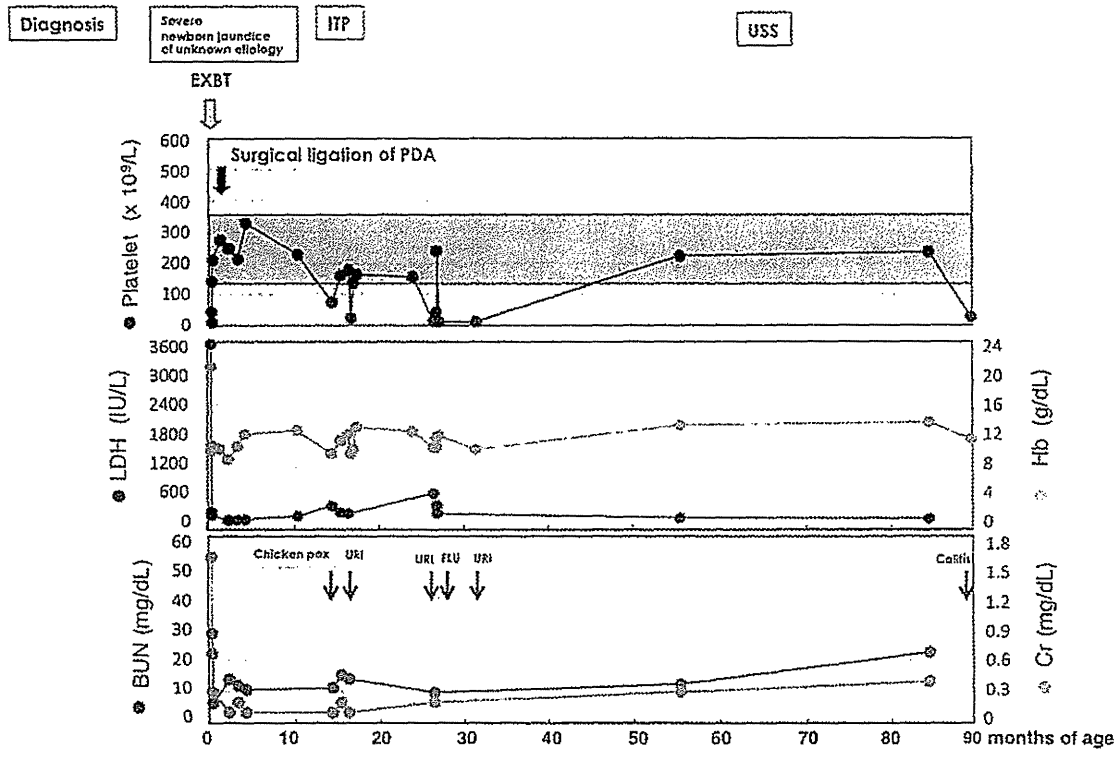


Fig. 3 Laboratory data and the clinical course of USS in patient HH4. In 2003, the proposita was born and transferred to Nihonkai General Hospital because she developed severe jaundice, cyanosis, and thrombocytopenia. After admission, she received exchange blood transfusions and platelet transfusions. At 14 months of age, she received a misdiagnosis of hemophagocytic syndrome associated with a viral infection. Since that time, she developed several episodes of petechiae with fever due to upper respiratory infection and influenza A. After she turned 3 years old, the incidence of petechiae decreased together with less frequent febrile episodes. In 2008, she received a diagnosis of USS based on severely deficient ADAMTS13 activity (<0.5 % of normal values) and no detected ADAMTS13 inhibitor. Interestingly, because her clinical signs and symptoms were mild, she did not receive FFP infusions. She is now 8 years old and has not developed major complications, such as renal insufficiency or neurologic abnormalities. *BUN* blood urea nitrogen, *Cr* creatinine, *Hb* hemoglobin, *PDA* patent ductus arteriosus, *EXBT* exchange blood transfusion, *ITP* idiopathic thrombocytopenic purpura, *USS* Upshaw–Schulman syndrome, *FLU* influenza A infection, *URI* upper respiratory infection

either prophylactically or in response to bouts of TTP [20]. On the other hand, patients categorized as having the late-onset phenotype develop the first episode of TTP after childhood. Patients with USS, however, occasionally show isolated mild thrombocytopenia during childhood, and, therefore, are overlooked or received a misdiagnosis of idiopathic thrombocytopenic purpura. These results indicate that USS with the late-onset phenotype may result from several different pathogenic processes. Generally, however, bouts of TTP in patients with USS are thought to be induced by various triggers, including pregnancy, upregulated cytokine expression during severe infections and excessive alcohol intake, among others [4].

Camilleri et al. [21] reported that a p.R1060W missense mutation in the ADAMTS13 gene was associated with USS with an ethnically specific late-onset phenotype; a Caucasian patient who was homozygous for the mutation showed plasma ADAMTS13 activity levels that were <5 % of normal values. Recently, we reported that a Japanese patient with USS was homozygous for a p.C1024R missense mutation, resulting in an Asian-specific late-onset phenotype. The patient received a correct diagnosis of USS at 77 years old and was shown to have moderately reduced plasma ADAMTS13 activity levels (2.4–3.4 % of normal values) using a sensitive chromogenic assay [13]. In vitro expression studies using HeLa cells transfected with plasmid encoding the p.C1024R mutant revealed that mutant protein was secreted into culture medium but at a significantly lower level than the wild-type protein. Further, the activity of the secreted p.C1024R mutant protein was three times that of the wild-type protein, indicating that p.C1024R was a gain-of-function mutation [22]. These data suggested that the plasma levels of ADAMTS13 activity in this patient prevented TTP during his childhood unless a strong stimulus-induced UL-VWFM release. Even in normal individuals, however, plasma levels of ADAMTS13 gradually decrease with age, in contrast to increasing plasma VWF levels [23]. Thus, in patients with USS, an

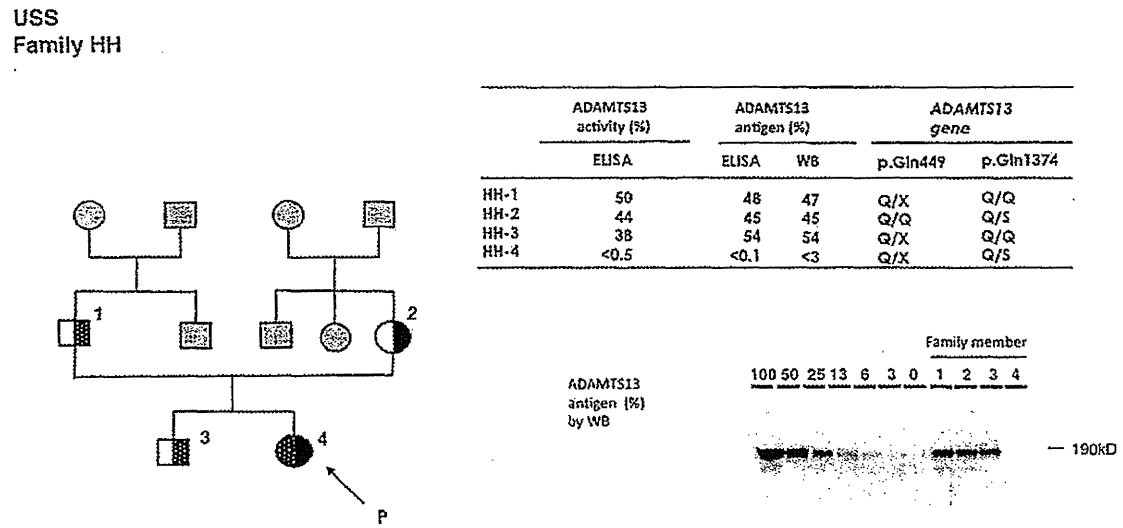


Fig. 4 Pedigree and ADAMTS13 analysis of USS-HH4 and her family. The proposita (denoted as P) USS-HH4 is the second of two offspring of non-consanguineous parents. Her parents and brother are asymptomatic carriers. ADAMTS13 activities were determined using activity ELISAs and ADAMTS13 antigen levels were measured using antigen ELISAs and Western blotting. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted as *one-letter amino-acid abbreviations* (*right upper panel*). Western blot analyses of plasma ADAMTS13 antigen in the patient's family members are shown in the *right lower panel*

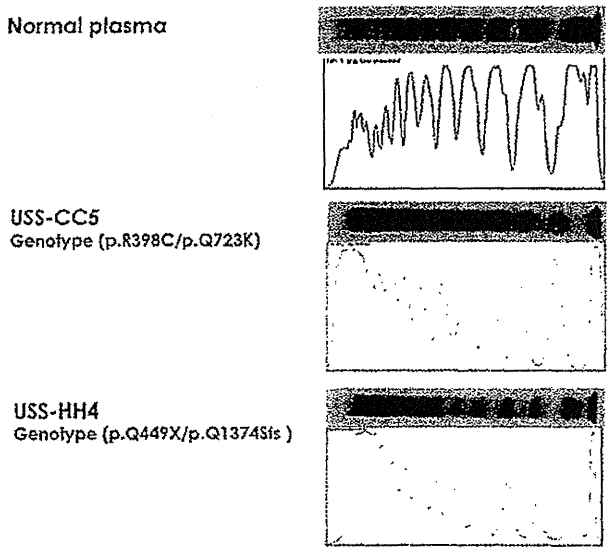


Fig. 5 Plasma VWF multimers from two patients with USS during remission phases. We analyzed VWF multimers in plasma samples obtained when the patients were in remission. Compared with the triplet bands observed in normal plasma (*upper panel*), VWF multimers in the patients' plasma samples consisted of single symmetrical bands (*middle panel* USS-CC5, *bottom panel* USS-HH4). Further, the patients' plasma samples showed high percentages of high-molecular-weight VWF multimers

imbalance in increased substrate levels (highly multimeric VWF) and the reduced ADAMTS13 enzymatic activity generates prothrombotic conditions, leading to more frequent TTP. Thus, mild or moderate deficiency of ADAMTS13 activity in patients with USS may contribute to conditions that allow the late-onset phenotype to develop.

Here, we have described two patients with USS (USS-CC5 and USS-HH4) who both had severe jaundice as newborns, requiring exchange blood transfusions. The subsequent clinical courses of disease in these patients, however, differed; USS-CC5 experienced chronic thrombocytopenia unless he was treated with prophylactic FFP infusions, whereas USS-HH4 developed transient thrombocytopenia only when she had an infection. USS-HH4 is now 8 years of age and has never been treated with FFP infusions. Notably, severe neonatal jaundice due to feto-maternal ABO incompatibility in USS-CC5—uncovered via an indirect positive Coombs test—masked a correct diagnosis of USS. ADAMTS13 gene analyses revealed that USS-CC5 and USS-HH4 were compound heterozygotes of p.R398C/p.Q723K and p.Q449X/p.Q1374Sfs, respectively. Among these mutations, p.Q449X was found in USS patients [16], but p.R398C, p.Q723K, and p.Q1374Sfs have not been previously reported in USS patient. Of these, p.Q723K alone was found as a rare nonsynonymous mutation in 128 normal individuals [6]. Both patients in this study showed plasma ADAMTS13 activity levels that were less than 0.5 % of normal, and ADAMTS13 antigen levels that were less than 0.1 % of normal. Therefore, it was suspected that p.R398C, p.Q723K, and p.Q1374Sfs were not secreted in plasma. Thus, the pathogenesis of the milder clinical presentation of USS-HH4 probably did not reflect the same mechanisms that contributed to the late-onset phenotype observed in the patient who was homozygous for the p.C1024R missense mutation. To determine whether UL-VWFMs were modulated by proteases other than ADAMTS13, we performed VWF multimer analysis

using patient plasma samples obtained during remission; each VWF multimer band in plasma from the two patients was represented by a single symmetrical band, rather than the triplet structure observed in normal plasma, indicating that VWF multimers from the patients had not been subjected to alternative proteolytic modifications.

In patient USS-HH4, the mechanism regulating the interactions between platelets and hyperactive UL-VWFMs without induction of overt TTP is presently unknown. Because we did not observe alternative proteolytic modifications of VWFMs in the patients with USS, we are now interested in potential fluid-phase regulatory mechanisms during high shear stress-induced platelet aggregation (H-SIPA). H-SIPA is dependent on VWFM size, and is inhibited by compounds that disrupt interactions in the VWF–platelet GPIb axis and subsequent platelet activation. Platelet activation during H-SIPA is mediated by endogenous ADP released from platelet δ-granules in microenvironments; therefore, ADP scavengers block H-SIPA without modifying VWFM structures. Indeed, we previously reported that human placental or vascular endothelial cell ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) efficiently inhibited H-SIPA [24]. Further, we also indicated the presence of soluble E-NTPDase in plasma, which is cleaved from the cell surface or generated by alternative splicing [25]. Thus, studies focusing on potential relationships between E-NTDase and H-SIPA would be of great interest, and may help to elucidate the pathogenesis of TTP in patients with USS.

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Conflict of interest Y. Fujimura is a member of clinical advisory boards for Baxter BioScience.

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The predictive value of anti-SS-A antibodies titration in pregnant women with fetal congenital heart block

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Abstract

Objective Fetal congenital complete heart block (CHB) is irreversible and is associated with significant mortality and morbidity. Anti-SS-A antibodies in the maternal sera are involved in its pathogenesis; however, the predictive value of the antibody titer and its role in prediction of this complication are controversial. The aim of this study was to determine the predictive value of maternal anti-SS-A antibodies on the development of fetal CHB.

Methods A retrospective chart review was performed for 189 cases of positive anti-SS-A antibodies determined by the double immunodiffusion (DID) method, and included 17 patients that developed fetal CHB. The relationship between the appearance of CHB and the anti-SS-A antibodies titer was examined.

Results An anti-SS-A antibodies titer of 1:32 or higher was identified by analyzing the receiver-operating characteristics (area under curve 0.72) curve. An anti-SS-A antibodies titer of 32 or more times greater than the upper limit by DID was a risk factor for fetal CHB (odds ratio 27.77, 95 % confidence interval (CI) 1.91–21.02, $P < 0.05$) in the multivariate analysis. Among 107 cases of anti-SS-A antibodies titers of 1:32 or higher, 65 patients (60.7 %) were treated with oral steroids. Of these, four patients had CHB (6.2 %). This rate of CHB was significantly lower ($P < 0.01$) than the rate in patients not treated with steroids.

Conclusion An anti-SS-A antibodies titer of 1:32 or higher in the maternal sera by DID was an independent risk factor for fetal CHB. In these patients, either antenatally administered prednisolone or betamethasone, was associated with a lower risk of fetal CHB.

Keywords Connective tissue diseases · Anti-SS-A antibodies · Pregnancy

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Introduction

Neonatal lupus erythematosus (NLE) is, in many cases a passively acquired autoimmune syndrome in which pathogenic autoantibodies (anti-SS-A antibodies) are transmitted from a mother to her fetus through the placenta. NLE is frequently associated with the presence of anti-SS-A antibodies in the mother [1].

Among the major clinical manifestations in infants with NLE, complete congenital heart block (CHB) is irreversible and requires the early implantation of a permanent

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pacemaker. In contrast, the non-cardiac manifestations are transient, resolving by 6 months of age without specific treatment [2]. CHB carries a significant mortality and morbidity including permanent pacing before adulthood [3]. The prevalence of CHB in children from women previously known to have anti-SS-A antibodies is ~1–2 % [4]. Thus, the prevention of CHB is an important issue in the management for pregnant women who test positive for anti-SS-A antibodies.

Several recent reports address the therapeutic approaches for fetal heart block. Findings of the PR interval and dexamethasone evaluation (PRIDE) study suggest that the PR interval should be measured regularly in fetuses at risk of heart block [5, 6]. Regular assessment of the fetal PR interval, however, may prove to be unduly burdensome for patients and physicians [7].

Transplacental steroid therapy has been proposed as a means of preventing CHB. Betamethasones are administered if the mother is positive for anti-SS-A antibodies, or has a history of a previous child with CHB. The efficacy of this, as well as that of dexamethasone, however, remains controversial [8]. Additionally, there is concern regarding the adverse neurodevelopmental effects of prenatal steroid exposure, thus a careful neurological assessment of fetuses treated with steroids is required [9].

The empiric treatment of all pregnant patients who test positive for anti-SS-A antibodies may subject an excessive number of fetuses to the detrimental effects of steroids. Therefore, a need exists for a means by which to identify the subset of fetuses at high risk of CHB who may benefit from steroids. While the 52 kD SS-A/Ro or 60 kD SS-A/Ro antibodies are clearly associated with CHB, other factors affect susceptibility [8]. For example, a prior history of CHB increases the risk ninefold, to 19 %, in subsequent pregnancies [4, 10]. Therefore, we reviewed the clinical courses of the patients in this study to identify other contributory factors, in addition to the anti-SS-A antibodies titer, that predicted the development of CHB.

Methods

Patients entered in this retrospective study were followed at one of five Japanese tertiary perinatal centers, including Kyushu University Hospital, Juntendo University, the University of Tsukuba, Osaka Medical Center and Research Institute for Maternal and Child, National Center for Child Health and Development, between 1996 and 2009. A total of 214 pregnant women with SS-A antibodies were enrolled in this study, and in 189 cases, anti-SS-A antibodies were titered by DID (double immune—diffusion) using commercially available kits (ENA-2 test, MBL, Nagoya, Japan or SRL, TFB, Tokyo, Japan). The correlation between these kits was verified by the supplier (personal communication).

Serum samples from 189 patients that were positive on an immunofluorescent screening test using HEp-2 cells were analyzed for anti-SS-A antibodies by DID in each laboratory using its current in-house methodology. The protocol numbers were: 21-71, 21-114, 22-610, 364 and 436 for Kyushu University Hospital, Juntendo University, University of Tsukuba, Osaka Medical Center and Research Institute for Maternal and Child, National Center for Child Health and Development, respectively. The study protocol opened for enrollment at each institution following approval by the ethics committee at each site.

The patients were divided into two groups based on whether the fetus developed CHB. A retrospective chart review was then performed to record maternal demographic characteristics, such as maternal age, parity, gestational week at delivery, frequency of premature delivery, deviation from standard birth weight, APGAR score (low APGAR at 5 min <7), antibody titer by DID, signs and symptoms of maternal autoimmune disease, and medications taken before and during the pregnancy. The deviation from the standard birth weight, a widely accepted method for evaluating the fetal growth, was calculated using the following formula: [(Mean weight at corresponding gestational week)-(actual BW)]/(Standard deviation at the corresponding gestational week) [11, 12].

Mean birth weight and the standard deviation at certain gestational ages were calculated using a formula derived from normal values for the Japanese population.

Multiple logistic regression and a receiver-operating characteristics (ROC) curve for levels of anti-SS-A antibodies by DID in the prediction of fetal CHB were calculated using EXCELL Tokei 2010—in Japanese (Shakai Joho Service, Tokyo, Japan). Statistical analysis was performed using the Mann-Whitney test, Chi-square test, and the unpaired t test programmed in GraphPad Prism® (GraphPad Software, Inc., CA). A P value of <0.05 was considered significant.

Results

Clinical profile

Mean age, parity, gestational week at delivery, frequency of premature delivery, birth weight, deviation from the standard birth weight, APGAR score at 5 min, cases with NLE, cases with CHB, in which the elevated anti-SS-A antibodies were demonstrated subsequent to the development of fetal CHB, signs and symptoms of maternal autoimmune disease, diagnosis of maternal autoimmune disease, and medications taken before and during the pregnancy are shown in Table 1.

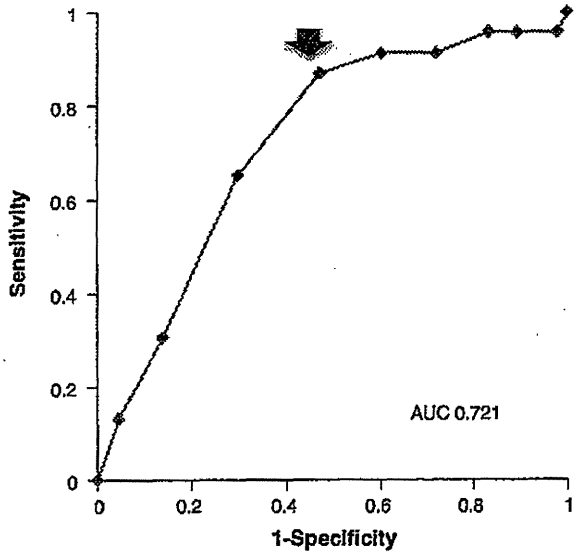

Fig. 1 Receiver-operating characteristics curve for the anti-SS-A antibodies titer in the prediction of fetal CHB *AUC* area under the curve, *arrow* denotes 32 times in DID

Multivariate analysis (Table 3)

We then performed a multivariate analysis using the seven variables significant to fetal CHB. As shown in Table 3, right panel, the odds ratio (95 % confidence interval) for maternal age, and an anti-SS-A antibodies titer of 1:32 or higher were 0.78 (0.62–0.98), $P < 0.05$; 27.77 (1.87–413.44), $P < 0.05$, respectively.

Efficacy of steroid therapy in patients with an anti-SS-A antibodies titer of 1:32 or higher (Tables 3, 4)

Use of steroids was not a predictor of CHB in the multivariate analysis. The association of steroid use with CHB was therefore assessed in the subset of patients with an anti-SS-A antibodies titer of 1:32 or higher (Table 3).

Among 107 patients with an anti-SS-A antibodies titer of 1:32 or higher, 65 (60.7 %) were treated with steroids taken orally during pregnancy; of these, four patients developed CHB (6.2 %). This percentage was significantly lower ($P < 0.01$) than that of the patients not treated with steroids. Among the patients treated with steroids, no patients (0 of 27) treated with prednisolone (dose: median 7.2 range 2.5–12.5 mg) and four of 38 patients treated with betamethasone (initiated at a dose of 2 mg/day at the gestational age of 12–20 weeks, with tapering after 2 weeks) developed CHB.

Fourteen of 41 patients had received prednisolone prior to receiving betamethasone, with the remaining 27 receiving betamethasone only.

Discussion

In this study we investigated risk factors for the development of CHB in anti-SS-A antibodies positive pregnant women. Our main finding was to establish anti-SS-A antibodies titer of 1:32 as the cut-off value based on analysis of a ROC curve. A multivariate analysis showed that an anti-SS-A antibodies titer of 1:32 or higher by DID was an independent risk factor for fetal CHB.

Franco et al. first described the relationship between maternal anti-SS-A antibodies and components of NLE, particularly CHB. Subsequently, several studies have investigated various antibodies including 52 kD SS-A/Ro or 60 kD SS-A/Ro, which do play significant roles. Little, however, is known regarding the relationship between the development of CHB and the anti-SS-A antibodies titer, and whether this relationship is causal.

The method used to titer the antibodies must be considered when assessing results. ELISA is commonly used given that it is simple to perform and automatable. False positive results, however, are common. The present study utilized DID. This method is the standard method for detecting anti-U1RNP, anti-Sm, anti-SS-A, anti-SS-B, anti-Scl-70, anti-Jo-1 antibodies, and is more reliable than the ELISA method [13].

Recently, in a study of 186 fetuses, Jaeggi et al. [8] identified fetal exposure to anti-SS-A levels ≧50 U/ml as significantly increasing the risk of CHB (5 vs. 0 % for <50 U/ml, odds ratio 7.8; range 0.4–159). This study employed enzyme-linked immunosorbent assay (ELISA) measurements. The ELISA assays utilized in the present and prior study were manufactured by different companies, which may explain the discrepancy in the results. Another recent study describes a standardized method for measuring both of the 52 and 60 kD SS-A antibodies, which is more sensitive and accurate than the conventional ELISA kits and the DID method [14, 15]. Thus, it is possible that further investigation using this new assay may confirm the predictive level of anti-SS-A antibodies by ELISA for CHB.

Another finding in this study was that antepartum steroid treatment with either prednisolone or betamethasone, may reduce the risk of fetal CHB in women with an anti-SS-A antibodies titer of 1:32 or higher. Use of either steroid significantly suppressed CHB in comparison to no treatment irrespective of which steroid was selected. Since orally administered prednisolone is inactivated by placental 11 beta HSD type 2 before reaching fetal heart [16], the effect of prednisolone is to diminish a generalized inflammatory insult and to eliminate the candidate maternal autoantibodies. Therefore, it is likely that the mechanism by which steroids affect CHB is maternal rather than fetal [17].

Table 3 Multivariate analysis for fetal CHB

	Odds ratio	95 % confidence interval	*P* value
Maternal age	0.78	0.62–0.98	**<0.05**
Gestational week at delivery	0.72	0.50–1.04	0.08
Apgar score (5 min)	0.83	0.48–1.42	0.49
Anti-SSA antibody titer 32 times or more	27.77	1.87–413.44	**<0.05**
Signs and symptoms of maternal autoimmune disease	0.86	0.13–5.62	0.88
Diagnosis of maternal autoimmune disease	0.27	0.05–1.48	0.13
Medications taken before and during pregnancy	0.2	0.04–1.06	0.06

Bold values indicate statistically significant

Multivariate analysis of seven predictors of fetal CHB was performed using EXCEL Tokei 2010—in Japanese (Shakai Joho Service, Tokyo, Japan). *P* values <0.05 were considered significant

DID double immunodiffusion

Table 4 Efficacy of steroid therapy in patients with an anti-SS-A titer 1:32 or higher

Steroid treatment	CHB		*P* value	
	Positive	Negative		
No steroids	12	30	<0.01	
Steroids, overall	4	62		
Prednisolone	0	25	<0.01*	0.082**
Bethamethazone	4	37	<0.05*	

* Comparison with patient without steroids

** Comparison between patients with prednisolone and betametazone

Autoimmune associated CHB occurs by a two-stage process. In the first step, maternal autoantibodies bind fetal cardiomyocytes, dysregulate calcium homeostasis, and induce apoptosis in affected cells. This step may clinically correspond to a first-degree heart block, and be reversible. As inflammation progresses, as may be the case in genetically susceptible fetuses, progressive tissue damage will lead to fibrosis, calcification of the AV-node and subsequent CHB [18]. It is plausible that the prevention of CHB is likely due, in part, to anti-inflammatory effects in the fetus. It is likely that suppression of the maternal autoimmune component also plays a role.

Patients with SLE or Sjögren Syndrome exhibit asymptomatic inflammation and fluctuations in the levels of numerous inflammatory cytokines [19]. NF-kappa B promotes a chronic inflammatory response through regulating the expression of genes involved in immunoinflammatory responses, cell cycle progression, inhibition of apoptosis, and cell adhesion [20]. These inflammatory processes may represent the target of prophylactic prednisolone in SLE mothers. Although the mothers with CHB fetuses were similar to the mothers without affected fetuses in terms of significant obstetrical history including a prior history of CHB in a fetus, it was not possible in the present study design to control for factors related to disease severity. Therefore, it is not possible from the present study to ascertain whether maternal disease modification by steroid treatment was directly related to a decreased risk of fetal heart block.

In our retrospective study, betamethasone was given to 41 patients with individual informed-choice base. Each patient was counseled with the understanding that not only has its efficacy in the prevention of fetal CHB not been established, but there are also possible adverse effects for the mother, including mood disorder, insomnia, and increased appetite, adverse obstetric events such as spontaneous abortion, stillbirth, neonatal adrenal insufficiency and long-term brain development. The protocol was not unified, but basically followed antecedent case reports [21, 22]. In brief, betamethasone was given from around 12 to 26 weeks of gestation, initiated at a dose of 2 mg/day, tapered every 2–4 weeks.

Transplacental steroid treatment carries potential risks. The major concerns with chronic steroid use are negative effects on neurological development, growth retardation, and oligohydramnios as well as hypertension, diabetes, infection, and osteonecrosis and osteoporosis in the mother. Fluorinated steroids have, in both human neonates and animal models, been shown to affect intrauterine growth and the central nervous system development with either single or repeated doses [18, 23]. It is unclear, however, whether the results of these studies are directly applicable to the fetus with CHB. Hutter et al. suggest that the risks of high-dose transplacental steroid treatment are in part avoidable by lowering the dexamethasone dosage [24]. As prednisolone was shown to have an equivalent effect in our study population, it is the preferable formulation as its adverse effects are generally considered acceptable even in early pregnancy [25]. A large, prospective study is necessary to ascertain the effectiveness and safety of prednisolone to prevent fetal CHB in patients with anti-SS-A antibodies titer of 1:32 or higher.

Table 1 Clinical profiles and comparison of outcomes between cases with or without fetal CHB

	Cases with CHB ($n = 17$)	Cases without CHB ($n = 172$)	P values
Age (years)*	30.2 (23.5 to 36.3)	33 (22 to 43.1)	**<0.05**
Gravidity*	0 (0 to 1)	0 (0 to 2)	0.94
Gestational week at delivery*	37 (32 to 39)	38 (29 to 41)	**<0.01**
Premature birth**	5 (29.4 %)	37 (21.5 %)	0.54
Birth weight (g)*	2300 (1172 to 3028)	2584 (948 to 3978)	0.05
Deviation from standard birth weight (SD)*	0.7 (−3.7 to 2.4)	−0.8 (−3.9 to 4.4)	0.90
Apgar score (5 min)*	8 (7–9)	9 (0 to 10)	**<0.01**
Previous child with CHB**	0 (0.0 %)	10 (5.8 %)	0.60
Anti-SS-A titer of 1:32 or higher	15 (88.2 %)	93 (54.1 %)	**<0.01**
Signs and symptoms maternal autoimmune disease**	8 (47.1 %)	131 (76.2 %)	**<0.01**
Diagnosis of maternal autoimmune disease**	8 (47.0 %)	137*** (79.6 %)	**<0.01**
Medications taken before and during	4 (23.5 %)	120 (69.8 %)	**<0.01**

Bold values indicate statistically significant

* Mean (range),** number of cases (1 %), + except for cases receiving medication after finding out fetal CHB, *CHB* congenital heart block, *NLE* neonatal lupus syndrome, *** includes seven asymptomatic patients diagnosed by chance, *DID* double immunodiffusion

Data are expressed as either the * median (range) or ** number of cases (%). The p values for the comparison between cases with fetal CHB versus cases without fetal CHB were calculated using either * Mann–Whitney test, ** Chi-square test (GraphPad Prism 5®, GraphPad Software, Inc., CA, USA). P values <0.05 were considered significant

Maternal autoimmune disease diagnoses and clinical details are presented in Table 2.

Predicting values (Fig. 1)

One hundred eighty-nine cases were available for evaluation with a receiver-operating characteristics (ROC) curve of the level of anti-SS-A antibodies (DID) and fetal CHB (morbidity). Based on the ROC curve at a cut-off point of 1:32 for the anti-SS-A antibodies titer, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 87.0, 53.0, 17.1, and 97.4 %, respectively, for predicting cases at high risk for CHB with an area under the curve (AUC) of 0.72 (Fig. 1).

Univariate analysis (Table 1)

A univariate analysis was performed analyzing the relationship between CHB and either maternal age, parity, gestational week at delivery, frequency of premature delivery, APGAR score at 5 min, and an anti-SS-A antibodies titer of 1:32 or higher, classification based on the ROC curve, presence of signs and symptoms of maternal autoimmune disease, diagnosis of maternal autoimmune disease, and medications taken before and during the pregnancy (Table 1).

Maternal age, gestational week at delivery, APGAR score at 5 min, an anti-SS-A antibodies titer of 1:32 or higher, presence of signs and symptoms of maternal autoimmune disease, diagnosis of maternal autoimmune disease, and medications taken before and during the pregnancy showed significant correlations.

Table 2 Clinical diagnosis of outcome between cases with or without fetal CHB

	Cases with CHB ($n = 17$)	Cases without CHB ($n = 172$)
Diagnosis of maternal autoimmune disease	8 (47.0 %)	137 (79.6 %)
Sjs	4	46
SLE	1	35
MCTD	0	4
APS	0	1
RA	**1**	**9**
Sjs/SLE	0	18
Sis/MCTD	1	3
Sjs/RA	0	5
Sjs/APS	0	1
SLE/APS	0	5
SLE/RA	1	0
SLE/MCTD	0	1
Sjs/SLE/APS	0	1
Sjs/SLE/RA	0	1
Others	0	7

Bold values indicate statistically significant

CHB congenital heart block, *NLE* neonatal lupus syndrome, *SLE* systemic lupus erythematosus, *Sjs* Sjögren syndrome, *RA* rheumatoid arthritis, *APS* Anti-phospholipid syndrome, *MCTD* mixed collagen tissue disease

In this study, we found that an anti-SS-A antibodies titer of 1:32 or higher by DID was an independent risk factor for fetal CHB. In these patients, either prednisolone or betamethasone, during pregnancy might reduce fetal CHB. These findings may provide a new clinical strategy to prevent fetal CHB in combination with PR measurements and conventional approaches.

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Conflict of interest None.

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