Alloantibodies against the B subunit of plasma factor XIII developed in its congenital deficiency

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Summary
Factor XIII (FXIII) is a fibrin-stabilising factor consisting of catalytic A subunits (FXIII-A) and carrier B subunits (FXIII-B). FXIII-B prevents the fast clearance of FXIII-A from the circulation. Congenital FXIII-A deficiency is a rare bleeding disorder, and congenital FXIII-B deficiency is even rarer. Through our recent nationwide survey on “acquired haemophilia-like disease due to anti-FXIII autoantibodies,” we newly diagnosed severe congenital FXIII-B deficiency in a Japanese man. He developed thrombocytopenia and gingival bleedings at the age of 73, and his FXIII activity was as low as 10% of the normal. When he suddenly developed spontaneous intramuscular haematoma, the bleeding was arrested by infusing FXIII concentrates. However, his FXIII activity remained around 10% of the normal. At the age of 74, ELISA and western blotting assay unexpectedly revealed complete absence of FXIII-B in the patient’s plasma. A dot blot assay detected anti-FXIII-B alloantibodies for the first time in this disease, which could be attributed to the infusion of exogenous FXIII. He was found to be homozygous for a Japanese founder-effect mutation of F13B. Repeated infusions of exogenous FXIII for hemostasis increased anti-FXIII-B alloantibodies that resisted FXIII substitution. To the best knowledge of the authors, none of the remaining 10 reported cases of congenital FXIII-B deficiency developed alloantibodies to exogenous FXIII-B of plasma FXIII. An originally mild bleeding phenotype of severe congenital FXIII-B deficiency can be exaggerated by additional acquired conditions. Physicians should consider congenital FXIII-B deficiency when they encounter cases of unexplained bleeding disorders.

Keywords
Factor concentrates, factor XIII/transglutaminases, inherited coagulation disorders, alloantibody, factor XIII-resistant state

Introduction
Coagulation factor XIII (FXIII) is a fibrin-stabilising factor, and is a heterotetramer consisting of two catalytic A subunits (FXIII-A) and two non-catalytic carrier B subunits (FXIII-B) (1-3).

Congenital FXIII deficiency causes a life-long severe bleeding tendency, abnormal wound healing, and recurrent miscarriages. It is a rare haemorrhagic disorder, and more than 500 cases of congenital FXIII deficiency have been identified throughout the world. In most of the reported cases, the congenital FXIII deficiency was caused by defects in the F13A gene (1, 4) (http://www.f13-database.de).

Only a few cases of congenital FXIII-B deficiency, caused by founder-effect mutations, have previously been identified in Japan and Italy (5-8). FXIII-B deficiency may be overlooked by physicians because of the mild bleeding symptoms of this condition (9). A homozygote with congenital FXIII-B deficiency has recently been reported from Germany (10). In an extensive literature survey, we found another reported case of severe FXIII-B deficiency in the USA (11).

During a recent Japanese nationwide survey on “acquired haemophilia-like disease (or haemorrhaphilia) due to anti-FXIII autoantibodies (AH13) (12, 13),” we newly diagnosed severe congenital FXIII-B deficiency (designated as FXIII-B Kurashiki) in a Japanese man, who developed alloantibodies, for the first time, against FXIII-B alloantibodies for the first time in this disease, which could be attributed to the infusion of exogenous FXIII. He was found to be homozygous for a Japanese founder-effect mutation of F13B. Repeated infusions of exogenous FXIII for hemostasis increased anti-FXIII-B alloantibodies that resisted FXIII substitution. To the best knowledge of the authors, none of the remaining 10 reported cases of congenital FXIII-B deficiency developed alloantibodies to exogenous FXIII-B of plasma FXIII. An originally mild bleeding phenotype of severe congenital FXIII-B deficiency can be exaggerated by additional acquired conditions. Physicians should consider congenital FXIII-B deficiency when they encounter cases of unexplained bleeding disorders.
The patient and his family did not have any history of bleeding tendency. At the age of 16 years, he had undergone left thoracic plastic surgery for pulmonary tuberculosis without any excessive bleeding. He started to experience repeated episodes of gingival bleedings at the age of 73 years, and he was tentatively diagnosed as immune thrombocytopenic purpura (ITP), because he showed purpura on the right hand, decreased platelet count (3.7 x 10^4/μl), increased immature platelet fraction (IPF; 19.8%), and increased levels of platelet-associated Immunoglobulin G (PAIgG; 146 ng/10^7 cells) in October 2009. Since then, he had been receiving treatment with prednisolone. When he started to manifest "delayed bleedings" after dental operations, he was administered an antifibrinolytic drug (trixamic acid). The results of the coagulation tests were within normal ranges, except for the FXIII activity and antigen levels (measured by a commercial laboratory, unless stated specifically) that were 12% and 9% of the normal, respectively, in December 2009 (Table 1, left column). Therefore, the amount of FXIII was moderately reduced but its antigen and activity levels were proportional (12/9=1.33; normal specific activity), suggesting the absence of FXIII inhibitor. His FXIII activity remained as low as 5-10% for more than six months (Figure 1). He then manifested gingival bleeding and leg purpura, and was infused with plasma-derived FXIII concentrates (FXIII Conc.; Lyophilised Human Blood Coagulation Factor XIII Concentrate, Fibrogammin P I.V. Injection, CSL Behring K.K., Tokyo, Japan) at 960 units (4 vials)/53 kg/day twice for haemostasis because of the persistent moderate FXIII deficiency. Since the bleeding symptom was observed to be abnormally severe than that expected to result from his decreased platelet count alone, his plasma and serum samples were extensively examined in January and February 2011 during our nationwide survey on AH13 (Table 1, right column).

In addition to thrombocytopenia, the patient developed anemia (Hb 9.9 g/dl) in June 2011, and 19 months after the first visit to our hospital, cytological examination of his bone-marrow aspirate indicated the myelodysplastic syndrome (REAB-1). The patient developed a spontaneous intramuscular haematoma in his right thigh, and he received FXIII Conc. at 24-hour (h) intervals for five days (Figure 1). He experienced repeated bleeding symptoms such as gingival bleeding and haemorrhoidal bleeding more frequently, and was transfused with FXIII Conc. for four days along with 10 units of platelet concentrates for both the bleeding events. Although his bleeding episodes were treated using appropriate amounts of FXIII Conc. (960 U/53.3 kg body weight [BW] = 18.0 U/kg), his plasma FXIII activities were as low as 7-9% 24 h after previous infusions and just before next infusions (8, 7, and 9% during the second, third and fourth bleeding episodes, respectively), and remained at the levels observed in the asymptomatic/non-bleeding periods (5-12%). Fortunately, until December 2012 (the time of submission of this manuscript) he was not clinically ‘refractory’ to FXIII replacement therapy, because his bleeding symptoms have been well controlled by infusing FXIII Conc.
Materials

Recombinant FXIII-A (rFXIII-A) was a kind gift from Dr. P. Bishop of Zymogenetics (Seattle, WA, USA). Recombinant FXIII-B (rFXIII-B) was expressed by baculovirus expression system and purified as previously described (14). Purified human plasma FXIII (A2B2 tetramer) was a generous gift from Dr. H. Kaetsu of Chaemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). An anti-FXIII-A monoclonal antibody was generously provided by Dr. G. Reed of Massachusetts General Hospital (Harvard Medical School, Boston, MA, USA). A rabbit anti-FXIII-A polyclonal antibody was generated in-house and affinity-purified using a rFXIII-A-coupled column. An anti-FXIII-B antisera was purchased from Nordic Immunological Laboratories (AX Eindhoven, The Netherlands), and its IgG was purified using Protein A-Sepharose (GE Healthcare Bioscience AB, Uppsala, Sweden) and biotinylated using ECL Protein Biotinylation Module (GE Healthcare, Waukesha, WI, USA). A tetrathymethylbenzidine (TMB) peroxidase substrate kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine thrombin, bovine serum albumin, N,N-dimethylcasein and monodansylcadaverine were purchased from Sigma-Aldrich (St. Louis, MO, USA). FXIII-deficient plasma was obtained from George King Bio-Medical (Overland Park, KS, USA).

Methods

This study was approved by the Institutional Review Board of the Yamagata University School of Medicine. All procedures were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all individuals including the present case and his family members.

Whole blood of the patients was collected into tubes containing 1/10 volume of 3.8% sodium citrate. Serum was separately prepared by using a tube containing glass microparticles to enhance coagulation. Plasma and serum samples were quick-frozen and compared by using a tube containing glass microparticles to enhance coagulation. Plasma and serum levels of FXIII-A antigen and FXIII-B antigen (15). The amount of plasma minus serum was incorporated assay

According to the Lorand’s method (16), 10 μl of plasma was incubated with 1 U bovine thrombin, 5 mM CaCl2, 20 mM Tris-HCl (pH 7.5), 0.2% N,N-dimethylcasein, 2 mM monodansylcadaverine, and 2 mM dithiothreitol in a 0.1 ml mixture at 37°C for 60 minutes (min). The reaction was terminated by adding 0.1 ml of 10% trichloracetic acid. The precipitate was collected by centrifugation, washed three times with 0.5 ml of ethanol-diethyl ether mixture (1: 1), and dissolved in 0.3 ml of 8 M urea, 1% sodium dodecyl sulfate (SDS) and 50 mM Tris-HCl (pH 8.0), which was abbreviated as UST buffer. The fluorescent intensity of emission at 520 nm with excitation at 360 nm was measured.

Fibrin cross-linking test

Ten microliters of plasma was mixed with 5 mM CaCl2 and 0.2 U bovine thrombin in a final volume of 20 μl, and incubated for the indicated time intervals. The reaction was terminated by adding 0.1 ml of 50 mM EDTA. The generated clot was collected by centrifugation at 10,000 g for 5 min, washed twice with 1 ml of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS), and dissolved in 40 μl of UST buffer, and then boiled with 40 μl of 2% SDS, 0.125 M Tris-HCl (pH 6.8), 15% glycerol, 5% β-mercaptoethanol, and 0.02% bromphenol blue (SDS-reducing buffer). A 10 μl sample was applied to SDS (0.1%)-polyacrylamide (10%) gel electrophoresis (PAGE) and proteins were stained with Coomassie brilliant blue R-250.

Western blotting

Plasma (1: 25, 1: 50, 1: 100 and 1: 200-dilutions with TBS) was boiled with 1% SDS, 50 mM Tris-HCl (pH 6.8), 7.5% glycerol, and 0.01% bromphenol blue in the presence (for FXIII-A) or absence

Table 1: Coagulation tests.

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<thead>
<tr>
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<tbody>
<tr>
<td>Platelet count</td>
<td>3.5 x 10^4/μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma α2-PI</td>
<td>102%</td>
<td>107%</td>
<td></td>
</tr>
<tr>
<td>Serum α2-PI</td>
<td>---</td>
<td>118%</td>
<td></td>
</tr>
<tr>
<td>XL-α2-PI</td>
<td>---</td>
<td>10.4%</td>
<td></td>
</tr>
<tr>
<td>XL-α2-PI ratio</td>
<td>---</td>
<td>0.081</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>commercial tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXIII activity</td>
<td>12%</td>
</tr>
<tr>
<td>1:1 cross-mixing</td>
<td>34% (control 128%)</td>
</tr>
<tr>
<td>FXIII antigen</td>
<td>9%</td>
</tr>
<tr>
<td>FXIII-A antigen</td>
<td>---</td>
</tr>
<tr>
<td>FXIII-B antigen</td>
<td>---</td>
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<tr>
<td>A2B2 antigen</td>
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Abnormal values are underlined. XL, crosslinked.
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Wada, Souri et al. First alloantibody against FXIII B subunit

(for FXIII-B) of 2.5% β-mercaptoethanol, and then applied to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked, and then reacted with an affinity-purified rabbit anti-human FXIII-A or anti-human FXIII-B antibody. FXIII antigens were detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and a chemiluminescent substrate.

Enzyme-linked immunosorbent assay (ELISA)

An anti-human FXIII-A monoclonal antibody was coated in a microtitre plate for the measurement of FXIII-A and Aβ2 complex, while anti-FXIII-B IgG was immobilised for measurement of FXIII-B. Plasma samples diluted 1/2,000 with TBS containing 2% bovine serum albumin were applied and incubated for 2 h at room temperature. After washing, for the determination of FXIII-A and Aβ2 complex, an anti-human FXIII-A polyclonal antibody and an anti-human FXIII-B antiserum, respectively, were added and incubated for 60 min. After washing, HRP-conjugated anti-rabbit IgG was added and incubated for 60 min.

For the determination of FXIII-B, biotin-conjugated rabbit anti-human FXIII-B IgG was added and incubated for 60 min. After washing, HRP-conjugated streptavidin was added and incubated for 60 min. After final washing, TMB was added, and the reaction was stopped after 10 min by adding 0.5 M H2SO4. Absorbance at 450 nm was recorded by a microtitre plate reader BioLumin 960 (Molecular Dynamics, San Diego, CA, USA) and compared to standard curves, using purified FXIII-A, Aβ2 complex, or FXIII-B.

Five-step cross-mixing test

In cross-mixing test, patient's plasma and normal control plasma were mixed at ratio of 0: 1, 1: 3, 1: 1, 3: 1, and 1: 0 and incubated at 37°C for 2 h, before amine-incorporation assay.

Dot blot assay

Fifty and 100 ng of either rFXIII-A or rFXIII-B, or 100 and 200 ng of rFXIII complex (Aβ2 tetramer) were blotted onto a nitrocellulose membrane, and reacted with the patient's plasma at a dilution of 1: 2,000. Immunoglobulin bound to either one of these FXIII antigens was detected using peroxidase-conjugated anti-human immunoglobulins (G+M+A, MP Biomedicals, Solon, OH, USA) and a chemiluminescent substrate (GE Healthcare).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

A Japanese founder-effect mutation of the F13B gene, a deletion of adenosine [ag- (-) g] or IVS1-2delA at the boundary of intron A/exon II, resulting in a loss of the obligatory AG splicing sequence, was detected as described previously (5, 6). PCR was carried out using a pair of gene-specific primers for intron A/exon II, 5’-TGCAGACCGGATATGCGAAGCT-3’ & 5’-TCCATG-TGTTCCTTTCTTACCGAAG-3’. An amplified product of 500 bp was treated with 5 U of TaqI endonuclease (Toyobo, Tokyo, Japan) at 65°C for 90 min, then the sample was applied to a 2% agarose gel.

Results and discussion

Diagnosis of FXIII-B deficiency

Through our recent nationwide survey on the incidence of AH13 (12, 13), we identified a new case of severe FXIII-B deficiency. Since the results of all the coagulation tests were within normal ranges for this elderly Japanese male patient, various minute examinations related to FXIII were carried out. Patient's plasma had a moderately low level of FXIII activity; 10% of the normal by a commercial ammonia release assay (Table 1, middle column). The difference between a2-PI activities in patient's plasma and serum (defined as XL-a2-PI) was also observed to decrease by a commercial assay, suggesting a reduced FXIII activity against its physiological substrates as well (13). In the patient's plasma obtained about one month after the commercial tests, his FXIII activity was 5% of the normal by our amine-incorporation assay. Results of our fibrin cross-linking test showed delayed γ-dimerisation as well as delayed α-polymerisation (Figure 2A). In addition, no band of the FXIII-B protein was detected in our western blot analysis (Figure 2B), which is consistent with the findings of our ELISA (<2% of the normal; Table 1, right column). These results clearly indicated the complete absence of FXIII-B antigen in the patient's plasma. He was diagnosed as congenital FXIII-B deficiency, as described later.

FXIII-A antigen was also barely detected by our western blotting analysis (Figure 2B) and <2% of the normal by our ELISA (Table 1, right column), indicating a secondary deficiency of FXIII-A due to the lack of protection by FXIII-B. In fact, FXIII-B prevents the rapid clearance of FXIII-A from the circulation in humans (11, 17) and mice (18).

Detection of anti-FXIII-B alloantibodies

Among our screening tests for detection of AH13 cases (13, 15), a cross-mixing test (1: 1) performed using the patient's plasma and normal control plasma with the commercial ammonia release assay showed moderate inhibition, because the resulting FXIII activity was 34% whereas the theoretical value was 69% [(10+128=138)/2] (Table 1, middle column). Moreover, his FXIII antigen level (45% by a commercial test) was 4.5 times higher than its activity (10%), suggesting the presence of inactive or inhibited FXIII molecules (10/45=0.22; low specific activity). After about one month, our five-step dilution cross-mixing test performed using our amine-incorporation assay showed a straight “deficiency” pattern at large (Figure 2C), which indicated a negative result for anti-FXIII inhibitor. However, our dot blot assays performed using rFXIII-A, rFXIII-B, and their complex clearly demonstrated the presence of anti-FXIII-B antibodies (Figure 2D). The reason for the discrepancy between the result...
of a commercial ammonia release assay and that of our amine-incorporation assay (about one month later) is unknown. It is possible that the majority of his anti-FXIII antibodies in January 2011 might be directed against A<sub>2</sub>B<sub>2</sub> tetramer, leading to inhibition of FXIII activation/activity.

It is highly likely that the anti-FXIII-B antibodies were caused by therapeutic infusion of plasma-derived FXIII Conc. containing exogenous FXIII-B as A<sub>2</sub>B<sub>2</sub> tetramer. The patient had been treated with prednisolone for suspected ITP. During the first period of the FXIII replacement therapy, the dose of prednisolone (7.5 mg/day) may not have been high enough to prevent the generation of alloantibodies against exogenous FXIII-B. As predicted, the patient's plasma clearly showed a drastic increase in anti-FXIII-B antibodies after three additional courses of the infusion with FXIII Conc. (Figure 3A and B).

The increase in anti-FXIII-B alloantibodies was confirmed by ELISA when FXIII Conc. was administered to the patient for arresting low gastrointestinal (GI) bleeding in July 2012 (Figure 3D), although the antibodies had been reduced beforehand probably by the immunosuppressive treatment with cyclosporine A (CsA) (Figure 1).

As of March 2012, only 11 cases (7 females and 4 males) of severe congenital FXIII-B deficiency have been identified on the basis of the absence of the FXIII-B antigen and the presence of defects in the F13B gene (9). Although seven cases with congenital FXIII-A deficiency were reported to have developed anti-FXIII-A alloantibodies (16, 19-25), no FXIII-B-deficient case with anti-FXIII-B antibodies has been described to date, to the best of our knowledge. Therefore, this is the first case of congenital FXIII-B deficiency in which the patient developed alloantibodies against exogenous FXIII-B.
‘FXIII-resistance’ to FXIII substitution by anti-FXIII-B alloantibodies

It is reported that a case with “acquired” FXIII-B deficiency due to anti-FXIII-B “autoantibodies” manifested severe bleeding symptoms (26). The presence of anti-FXIII-B ‘alloantibodies’ may also have clinical implications on the patient condition at least to some extent, every time when he undergoes FXIII replacement therapy. When the patient was infused with FXIII Conc. for arresting the bleeding, the antibodies may have bound to the exogenous FXIII and the resultant antigen-antibody complexes may have been rapidly removed from the circulation. This assumption was consistent with the fact that his FXIII activity increased only by 2-4% from the lowest basal level of 5%, even though the estimated increase in the activity after the infusion of FXIII Conc. was about 35% [960x2 U/53kg BW]. The patient’s plasma FXIII activities remained at 7-9% 24 h after previous FXIII Conc. infusions. This state is, as it were, pharmaceutically ‘FXIII-resistance’.

To test this assumption further, we measured FXIII activity immediately after infusion of FXIII Conc. and estimated its half-life in the patient when he manifested GI bleeding in July 2012 (Figure 4A). The increase in FXIII activity was only partial (10% measured vs. 1,200x2 U/50 kg BW = 48% calculated). Since the patient’s FXIII activity returned to his baseline level after 12 h, the half-life was roughly estimated to be about 3 h. The half-life for the same plasma-derived FXIII Conc. (Fibrogammin P) was reported as about 9 h in a Hispanic congenital FXIII-B deficient case (11), while it was 17 h in the Hungarian case with ‘acquired’ FXIII-B deficiency due to anti-FXIII-B autoantibodies (26). Thus, the extremely short half-life of the present case was very likely due to a combination of the complete absence of FXIII-B and the presence of anti-FXIII-B alloantibodies. The preceding infusion of FXIII concentrates may have boosted the production of anti-FXIII-B alloantibodies again, because essentially no increase in FXIII activity was observed after next infusion of 1,200 U FXIII Conc. for his gingival bleeding after three months (Figure 4B).

The exogenous FXIII may have functioned transiently and/or locally at the sites of bleeding even though his systemic FXIII level was not confirmed to increase significantly. The alloantibodies against FXIII-B should be eradicated by immunosuppressive therapy (15) to improve his pharmacological FXIII-resistant state, if he continues to experience severe bleeding episodes, which may finally become clinically refractory to FXIII replacement therapy. Because he had already been treated with cyclosporine A since April 2012, other regimens such as cyclophosphamide and rituximab, as used for autoimmune FXIII deficiency (26), may be more successful in the eradication therapy. In addition, replacement therapy using a commercial recombinant FXIII-A preparation will be a useful and reasonable option to avoid further boosting the production of his anti-FXIII-B alloantibody, when it will be licensed in Japan in the near future.

Family study and identification of founder-effect mutation

The results indicating the patient’s severe FXIII-B deficiency prompted us to carry out a family study as well as genetic analyses of his pedigree. In the two daughters of the proband (FXIII-B Kur-
ashiki), ELISA results clearly confirmed decreased amounts of both FXIII-B (39 and 45% of normal, respectively) and FXIII-A (33 and 59% of normal, respectively) proteins (Figure 5A), which was consistent with the results of western blotting analysis (data not shown). Both daughters showed FXIII amine-incorporation activity of 38 and 74% of the normal, respectively. A commercial ammonia release assay also demonstrated similar results (39 and 70% of the normal, respectively). It is interesting to note that about two thirds of 37 defined heterozygotes of congenital FXIII-B deficiency showed decreased FXIII activity and antigen levels (see Suppl. Table 1, available online at www.thrombosis-online.com), suggesting that more than a half of the normal FXIII-B level may be necessary to maintain/support normal FXIII/FXIII-A levels in the majority of these individuals. Since the concentration of free FXIII-B is constant regardless the concentration of FXIII-A or $A_2B_2$ tetramer (27), only a part of the reduced FXIII-B by half may complex with FXIII-A to stabilise it (10).

We previously identified a founder-effect mutation in the $F_{13}B$ gene among Japanese individuals (5, 6). This deletion mutation was detected by PCR-RFLP analyses in the homozygous and heterozygous states in the genomic DNA of the proband (III-3) and his daughters (IV-1 & -2), respectively (Figure 5B). Since the same mutation was observed in individuals from four geographically separated regions of Japan, Kanazawa, Fukuoka, Fukushima and Kurashiki (see Suppl. Table 1, available online at www.thrombosis-online.com), we believe that there would be many more patients with this type of congenital FXIII-B deficiency, at least in Japan. As recently addressed (9), bleeding symptoms of these Japanese patients seem to be mild; spontaneous bleedings rarely develop while provoked bleedings secondary to haemostatic challenges, such as surgery, trauma, and delivery in females are common.

In conclusion, congenital FXIII-B deficiency seems to be more common than what we perceived previously. Although the bleeding phenotype in congenital FXIII-B deficiency is naturally mild, severe bleeding episodes can occur when the condition is aggravated by additional haemostatic disorders, including the development of thrombocytopenia resulting from myelodysplastic syn-
drome and FXIII-resistance by anti-FXIII antibodies etc., like the present case. Thus, physicians need to be aware of congenital FXIII-B deficiency, and they must keep this disease in mind whenever they come across patients showing unexplained bleeding disorders.

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Conflicts of interest
None declared.

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