Rapid immunochromatographic test for detection of anti-factor XIII A subunit antibodies can diagnose 90% of cases with autoimmune haemorrhaphilia XIII/13

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Summary
Autoimmune haemorrhaphilia XIII/13 (AH13) is an acquired life-threatening bleeding disorder due to anti-factor XIII (FXIII) autoantibodies (auto-Abs). AH13 patients may die of haemorrhage without correct diagnosis and proper treatment because of lack of awareness and the absence of rapid easy-to-use tests specific for this disease. Currently, the definitive diagnosis is established by cumbersome and time-consuming laboratory tests such as dot-blot assays and enzyme-linked immunosorbent assays (ELISA), and therefore these tests are generally not carried out. To save AH13 patients’ lives, there is an urgent necessity for developing a rapid test for FXIII auto-Abs. We first generated and characterised mouse monoclonal antibodies (mAb) against human FXIII A subunit (FXIII-A), and then developed a rapid immunochromatographic test (ICT) for detection of anti-FXIII-A auto-Abs using one mAb with a dissociation constant of $9.3 \times 10^{-11}$ M. The auto-Ab-FXIII-A complex was captured by the mAb on a nitrocellulose membrane and visualised by Au-conjugated anti-human IgG Ab. Mixing with healthy control plasma improved the detection of auto-Abs in patients having extremely low levels of FXIII-A. The specificity and sensitivity of the ICT were 87% and 94%, respectively. We also detected auto-Abs against activated FXIII (FXIIIa) in three patients by pre-converting FXIII to FXIIIa by thrombin treatment. ICT values were significantly inversely correlated with FXIII activity levels, indicating an association between the quantity of anti-FXIII autoantibodies and AH13. This reliable rapid ICT assay can be applied to a point-of-care test to detect anti-FXIII-A auto-Abs, and will contribute to early diagnosis and treatment of AH13.

Keywords
Acquired coagulation disorders, autoantibodies, autoimmune diseases, factor XIII / transglutaminases, diagnosis management

Introduction
Factor XIII (FXIII) is a plasma pro-transglutaminase consisting of a catalytic A subunit (FXIII-A) dimer and a carrier B subunit (FXIII-B) dimer that circulates in the blood as a heterotetramer. During the final stage of blood coagulation, FXIII is activated by thrombin and Ca$^{2+}$, and the resultant activated FXIII (FXIIIa) crosslinks the $\gamma$-glutamyl-$\epsilon$-lysine residues in fibrin and the $\alpha_2$-plasmin inhibitor as well as fibrin monomers to form stable fibrin clots with increased resistance to mechanical stresses and fibrinolysis (1–4).

Activated FXIII deficiency is mainly caused by a secondary FXIII reduction via hypo-synthesis and/or hyper-consumption due to a primary disease(s) (4, 5). However, anti-FXIII autoantibodies (auto-Abs) cause acquired haemophilia-like disease (or haemorrhha-philia; termed AH13 in this manuscript), which manifests more severe bleeding symptoms than non-autoimmune haemorrhagic acquired FXIII deficiency (AFD) (5, 6).

Acquired FXIII inhibitors, especially anti-Abs, are classified into three major types: type I inhibitors that prevent the activation of FXIII (7–10); type II inhibitors that interfere with the transamidase activity of FXIIIa; and type III inhibitors that are directed against the fibrin itself, blocking the crosslinking sites for access to FXIIIa. This classification applies to anti-fibrin auto-Abs but not to anti-FXIII-B auto-Abs (11, 12). Therefore, we recently proposed classifying anti-FXIII auto-Abs into types Aa, Ab, and B by their immunological properties, i.e. directed against FXIII-A, FXIIIa, and FXIII-B, respectively (unpublished data). As of April 2014, approximately 80%, 10%, and 10% of our 32 AH13 patients belonged to types Aa, Ab, and B, respectively.

AH13 is thought to be rare (13, 14). However, the number of patients has recently been increasing in Japan (15), probably because Japan has become a so-called “super-aging” society. In fact, we have diagnosed 44 Japanese AH13 cases during the last 11 years (as of October 2014; unpublished data), while only eight Japanese AH13 cases were reported by other researchers before 2000, to the authors’ best knowledge. In addition, 17 non-Japanese AH13 cases were documented in the last century (4, 13). AH13, however, is not well known even among Japanese physicians and some patients never receive the correct diagnosis and proper treatment.
Many patients with AH13 are considered to have an unexplained bleeding disorder because decreased FXIII activity cannot be detected by routine coagulation tests such as the prothrombin time and activated partial thromboplastin time tests.

The definitive diagnosis of AH13 can currently be established only through time-consuming and expensive laboratory tests, such as the dot-blot assay and enzyme-linked immunosorbent assay (ELISA) that are carried out in a limited number of medical facilities. Therefore, a rapid point-of-care test (POCT) (16–19) for AH13 is necessary because a prompt differential diagnosis between AH13 and AFD is essential for proper treatment.

In this study, we generated and characterized mouse monoclonal antibodies (mAbs) against human FXIII-A, and developed an immunochromatographic test (ICT) that rapidly detects anti-FXIII auto-Abs to diagnose AH13.

Materials and methods

Materials

Recombinant FXIII-A (rFXIII-A) was kindly provided by Zymogenetics (Seattle, WA, USA). Recombinant FXIII-B (rFXIII-B) was expressed by the baculovirus system and purified as previously described (20). Anti-FXIII-A and anti-FXIII-B polyclonal antibodies (pAbs) were purchased from Calbiochem (San Diego, CA, USA) and each immunoglobulin G (IgG) was purified and biotinylated as previously described (21). A horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-streptavidin, Protein G-Sepharose, and CNBr-activated Sepharose 4B were obtained from GE Healthcare Bioscience AB (Uppsala, Sweden). A Tetramethylbenzidine (TMB) Peroxidase Substrate Kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine chymotrypsin, thrombin, and Gly-Pro-Arg-Pro(GPRP)-NH₂ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Wako Pure Chemical Ind. (Osaka, Japan). FXIII-A peptides were synthesised by Sigma-Genosys (Hokkaido, Japan).

Production of in-house mouse mAbs against human FXIII-A

Female six-week-old BALB/c mice were subcutaneously immunised every two weeks three times with purified human plasma-FXIII emulsified in complete Freund’s adjuvant. Four days after intraperitoneal booster injections of FXIII, the mouse splenic cells were fused with NS-1 myeloma cells. Hybridomas producing a large quantity of anti-FXIII mAbs were screened for their ability to bind to purified FXIII by a sandwich ELISA using a rabbit anti-mouse γ-globulin antibody. After cloning by limiting dilution several times, the selected cell lines were cultured, and monoclonal antibodies (mAbs) were purified from culture supernatants by 40% ammonium sulfate precipitation and gel-filtration using a Sephacryl S-200 column after digestion with pepsin.

ELISA

ELISA was performed as described previously (21) with several modifications. IgGs of anti-FXIII-A and anti-FXIII-B pAbs were coated for the measurement of FXIII-A and FXIII-B, respectively. Three doses (1, 5, and 25 ng) of rFXIII-A or rFXIII-B were then applied and incubated, followed by incubation with in-house mAbs (1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E; 3.3 nM each).

To determine the dissociation constant ($K_d$) of in-house mAbs and rFXIII-A, ELISA was performed with increasing concentrations (0.07–4.3 nM) of the mAbs and a fixed concentration (5 ng, 0.6 nM) of rFXIII-A. Double reciprocal plots of the ELISA signal versus the concentration of the mAbs were evaluated. A $K_d$ value for each mAb was determined as previously described (22).

Dot-blot analyses

Denatured rFXIII-A was prepared by boiling rFXIII-A in 125 mM Tris-buffer (pH 6.8) containing 0.1% SDS. Native and denatured rFXIII-A (1, 10, and 100 ng) was spotted on nitrocellulose membranes. A dot-blot analysis was performed as previously described (23). One of the mAbs against FXIII-A and HRP-conjugated anti-mouse IgG was used for the primary and secondary antibodies, respectively.

The effect of mAbs on the FXIII activation or FXIIIa activity

To detect the inhibitory effects of mAbs against FXIII activation or FXIIIa activity, rFXIII-A (100 ng) was pre-incubated with mAbs (5 µg) at 37°C for 1 hour (h) or thrombin (1 U) at 37°C for 15 minutes (min), and then incubated with thrombin (1 U) at 37°C for 15 min or mAbs (5 µg) at 37°C for 1 h.

A standard amine-incorporation (AI) assay was performed using 5 mM CaCl₂, 0.2% N,N-dimethylcasein, 2 mM monodan-sylcadaverine and 1 U bovine thrombin as previously described (8, 10, 21).

The effect of mAbs on the formation of the FXIII-A₂B₂ heterotetramer

Four doses (0.2, 1, 5, and 25 µg) of mAbs were incubated with rFXIII-A (1 µg) in 2% bovine serum albumin overnight at 4°C and subsequently incubated with rFXIII-B (1 µg) in 20 µl for 20 min on ice. The FXIII-A₂B₂ heterotetramer was detected by ELISA as previously described (21).

Proteolytic digestion and western blot

Proteolysis of rFXIII-A (2 mg/ml) with trypsin (using an enzyme-to-substrate ratio E/S 1/500, w/w) was carried out at 37°C for 30 min in 20 mM Tris-buffered saline (TBS), pH 7.5 containing 10 mM CaCl₂. A western blot of the digested product was performed as previously described (23) with several modifications. One of the mAbs against FXIII-A and HRP-conjugated...
anti-mouse IgG was again used for the primary and secondary antibodies, respectively.

**Epitope analysis using FXIII-A peptide-coated plates by ELISA**

Microtitre plates were coated with FXIII-A peptides (2.5 nmol; Suppl. Table 1, available online at www.thrombosis-online.com) in 100 μl of 50 mM carbonate buffer (pH 9.6) overnight at 4°C. The plates were incubated with in-house mAbs (500 ng), and subsequently incubated with HRP-conjugated anti-mouse IgG. The reaction with the TMB substrate and the termination of the reaction were performed as described.

**Epitope analysis using mass spectrometry-based identification of digested fragments bound to each mAb**

Each anti-FXIII-A mAb-Sepharose (1.2 mg mAb/ml) column was prepared by coupling CNBr-activated Sepharose 4B with the in-house mAb according to the manufacturer’s protocol. The rFXIII-A was then incubated with mAb-Sepharose at 4°C for 2 h, followed by digestion using chymotrypsin (E/S = 1/100) at 37°C for 6 h. After washing with TBS containing 0.1 % Tween 20 and deionised water, bound peptides were eluted by 0.1 M glycine buffer (pH 2.5), neutralised by 1.5 M Tris-buffer (pH 8.8), and subjected to carboximidomethylation with iodoacetamide. For mass spectrometry (MS) identification, the samples were desalted using a C-Tip (Nikkyo Technos, Tokyo, Japan) according to the manufacturer’s protocol. To determine the ionisation efficiency of each peptide, rFXIII-A without mAbs was also digested using chymotrypsin, and carboximidomethylation and desalting were performed as described above.

**Nanoflow liquid chromatography-tandem MS (nanoLC-MS/MS)**

The desalted peptide solution was analysed by nanoLC-MS/MS as described previously (24) with several modifications, using the EASY-nLC 1000 system (Thermo Scientific, Hudson, NH, USA) on a nano-capillary column (NTTC-360/75–3, Nikkyo Technos). The nanoflow system was connected to a Quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a nanoelectrospray emitter. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. The full-scan spectra (m/z range 350–1800) were acquired. The 10 most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation (25) at a normalised collision energy of 28%.

**Peptide identification**

Raw file reads were searched against the Swiss-Prot human database (542,503 sequences) using Proteome Discoverer (version 1.4, Thermo Scientific) with the Sequest HT and Mascot (version 2.3, Matrix Science, Tokyo, Japan) search engines. Precursor and fragment mass tolerances were set to 5 ppm and 0.02 Da, respectively. Cysteine carboximidomethylation was set as a static modification. The results were filtered using Percolator with a false discovery rate of 1%. The peak area of each identified peptide was estimated using Proteome Discoverer.

**Clinical samples**

The authors consulted physicians in charge of unexplained haemorrhagic patients (14, 15). From June 2009 to July 2014, patients with severe bleeding who did not have a personal or family history of bleeding or abnormal clotting times were recruited for this study. Control plasma samples were obtained from healthy volunteers. 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.

**ICT for the detection of anti-FXIII-A auto-Abs**

The entire ICT procedure is summarised and an example is shown in Suppl. Figure 1 (available online at www.thrombosis-online.com). The in-house mAb, 1TH2–8C4C, was dispensed onto a nitrocellulose strip (test line). Patient or healthy control plasma samples (1:10 dilution) were applied to a 96-well microtitre plate. The strip was then inserted into a well for 5 min at 37°C, and after three washes, the strip was immersed in a solution containing the anti-human IgG Ab-gold conjugate for 15 min at 37°C. Reactions were read using a reader device (FactScan, Denken Co., Ltd., Oita, Japan) and expressed as an absorbance unit relative to that of a plasma sample from an AH13 patient (AH13-Aa-1) (assigned as 1 arbitrary unit, AU).

**Spiked ICTs**

Equal volumes of patient and commercially available pooled normal plasma (Sysmex, Kobe, Japan) were mixed and incubated at 37°C for 2 h prior to ICT (termed spiked ICT). To this reaction mixture, an equal volume of thrombin (0.01 U) in TBS containing 10 mM CaCl₂ with or without 1 mM GPRP-NH₂ was added and incubated at 37°C for 2 h. Samples were then diluted 5-times with a dilution buffer for ICT.

**Comparison of ICT and conventional dot-blot analysis**

Conventional dot-blot analyses of clinical samples for the detection of anti-FXIII-A auto-Abs were performed as previously described (23). The sensitivity of the dot-blot analysis and direct and spiked ICTs was calculated from the results of 16, 15, and 16 samples from patients with AH13, respectively. The specificity of these tests was calculated from results of 23 patients with AFD. To assess the agreement between direct or spiked ICT and the dot-blot analysis, the kappa coefficients were calculated as previously described (26).
Statistical analysis

For statistical analysis, values were expressed as mean ± standard deviation. Comparisons between groups were performed using Kruskal-Wallis tests of SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC, USA). Differences were considered significant at p-value of <0.05. Receiver operating characteristic (ROC) curve was used to determine the optimal cutoff values and the areas under curve (AUC) for ICT data.

Results

Characterisation of mouse mAbs against human FXIII-A

We first characterised three in-house mAbs for use in development of an ICT for detection of anti-FXIII-A auto-Abs; all mAbs readily detected native rFXIII-A at concentrations of 10 and 100 ng by dot-blot analyses (data not shown). Denatured rFXIII-A, however, was detected unambiguously only at 100 ng. Therefore, these mAbs recognized native rFXIII-A approximately 10 times more efficiently than denatured rFXIII-A.

All of the three mAbs bound to rFXIII-A in a dose-dependent manner in ELISA (Figure 1A). We also determined a K_d value for each mAb against FXIII-A using double reciprocal plots of the ELISA signal versus the concentration of mAbs (Figure 1B). The plots were linear and the K_d values of 1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E were 9.3 × 10^{-11}, 1.4 × 10^{-10}, and 1.2 × 10^{-10} M, respectively.

When these mAbs were pre-incubated with rFXIII-A prior to thrombin activation, AI activities in the presence of 1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E were 116 ± 5, 117 ± 9, and 122 ± 14% (n = 3), respectively. These values were slightly higher compared with that obtained in the absence of mAbs (assigned as 100%). Because we did not inactivate thrombin, mAbs may have protected rFXIII-A from extensive digestion of rFXIII-A by thrombin (incubated for a total of 45 min) and its consequent degradation.

We also tested whether mAbs inhibit FXIIIa pre-activated by thrombin. AI activities of FXIIIa in the presence of 1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E were 174 ± 3, 160 ± 3, and 183 ± 11% (n = 3), respectively. These values were considerably higher compared with the values obtained in the absence of mAbs (100%) (incubation for a total of 1 h and 45 min). These results indicate that the mAbs do not inhibit either FXIIIa activity or FXIII activation. In addition, all three mAbs may bind near the FXIIIa interaction site between the core and barrel-1 domains.

Finally, the heterotetramer formation was in the presence of 0.2, 1, 5, and 25 µg of 1TH2–8C4C, 82, 71, 134, and 101 %, respectively, compared with that in the absence of mAbs (100%). The heterotetramer formation was 91, 116, 137, and 87% in the presence of 1TH6–2H7F, and 121, 117, 83, and 107% in the presence of 1TH6–10E, respectively. These results indicate that the three mAbs do not inhibit FXIII-A_{B2} heterotetramer assembly.

Epitope analysis of each mouse mAb

Western blot analysis of trypsin-digested rFXIII-A was performed to determine the approximate regions recognised by mAbs. There were three major bands, 24, 54, and 76 kDa visualised by Coomassie staining, corresponding to the barrel-1 and -2, β-sandwich and core, and β-sandwich and core and barrel-1 and -2 domains, respectively. However, the 24 and 76 kDa bands, but not the 54-kDa band, were detected by all three mAbs, indicating that all three mAbs recognise the barrel-1 or barrel-2 domain.

We used 35 synthesised peptides with sequences covering 83% of the FXIII-A molecule to localise the epitope(s) of each mAb (Suppl. Table 1, available online at www.thrombosis-online.com). All mAbs bound to the FXIII-A-peptides (P) 3, 5, 26, 27, 28, and 29 coated on plates and generated strong ELISA signals over 0.05 (absorbance at 450 nm, Suppl. Figure 2A, available online at www.thrombosis-online.com). There were some differences in the binding intensity to the peptides between these mAbs, e.g. 1TH2–8C4C bound to P5.
and P28, 1TH6–2H7F to P27 and P29, and 1TH6–10E to P3 and P26 more strongly than the other two mAbs, respectively. These results suggested that the epitopes of the mAbs were organised as epitopic mosaics distributed around the β-barrel-1 and/or β-sandwich domains of the FXIII-A molecule, and thus these might be discontinuous ‘structural/conformational’ epitopes.

Epitope mapping of each mouse mAb by MS of enzyme-digested fragments

To further localise the mAbs epitopes rFXIII was digested with chymotrypsin, and the rFXIII-A-derived fragments bound to each mAb were identified using MS. Chymotrypsin digestion produced 79, 58, and 56 fragments of rFXIII-A bound to 1TH2–8C, 1T6–2H7F, and 1TH610E, respectively, and of these nine, seven, and six major fragments were detected, respectively, with peak areas of over 10\(^6\) (1.000E9), that originated from the β-sandwich and barrel-1 domains of FXIII-A (Suppl. Figure 2B and Suppl. Table 2, columns 11–13 highlighted, available online at www.thrombosis-online.com). In contrast, among 80 chymotrypsin-digested fragments derived from the same amount of rFXIII-A alone (Total), 54 fragments had peak areas of more than 10\(^5\) and originated from domains throughout the entire FXIII-A molecule (Suppl. Figure 2B and Suppl. Table 2, total area in column 14 highlighted, available online at www.thrombosis-online.com).

The relative peak areas of the major mAb-bound fragments to the Total areas (Suppl. Table 2, column 14, available online at www.thrombosis-online.com) were estimated (in parentheses). The estimated relative area of FXIII-A-(amino acid numbers 145–157), -(581–588), -(581–594), and -(607–619) bound to 1TH2–8C4C, those of FXIII-A-(581–588) and -(607–619) bound to 1TH6–2H7F and 1TH6–10E, and that of FXIII-A-(581–594) bound to 1TH6–2H7F were more than 0.5.

These results clearly indicated that the epitopes of all three mAbs were located primarily in the β-barrel-1 domain.

Development of a direct ICT using the in-house mAb

We developed an ICT for detection of anti-FXIII-A auto-Abs using 1TH2–8C4C because it demonstrated the highest affinity to rFXIII-A among the three mAbs. We first carried out 20 trial runs of a healthy control plasma sample on different days to assess the reproducibility of the ICT. The mean and standard deviation (SD) of the test line signal intensity were 0.09 and 0.03 AU, respectively.

We then determined a mean and SD using the plasma of 24 healthy controls to establish a cut-off value of the test (Figure 2A). The mean and SD of the test line intensity were 0.08 and 0.05 AU, respectively. We therefore established the cut-off value of 0.18 AU for the present ICT using the mean plus 2 SDs.

Evaluation of the direct ICT for detection of anti-FXIII-A auto-Abs in clinical samples

We performed the ICT on plasma samples from 38 AH13-suspected patients to determine whether anti-FXIII-A auto-Abs were present. As a result, 12 samples yielded a positive reaction in the ICT, while 26 samples yielded a negative reaction (Figure 2B and C). The conventional dot-blot assay used for definitive diagnosis classified 14 samples as positive for anti-FXIII-A auto-Abs and 24 samples negative (Suppl. Table 3, available online at www.thrombosis-online.com).

One (AFD-10) of the 12 positive samples assessed using ICT was considered to be a false positive because of the negative results obtained with the dot-blot assay and cross-mixing tests (data not shown). However, four of the 26 negative samples determined by ICT were false negatives. Therefore, the specificity of the ICT to detect anti-FXIII-A auto-Abs was 96% (22/23), while the sensitivity was 73% (11/15). A kappa coefficient of 0.77 was in the “good to fair” agreement range between the ICT and dot-blot assays.

Evaluation of spiked ICT for detection of anti-FXIII-A auto-Abs

Several patients with AH13 have extremely low levels of FXIII-A, and therefore the quantity of a quadripartite complex consisting of mAb (1TH2–8C4C), FXIII-A, auto-Abs, and anti-human IgG Ab-gold conjugate is not sufficient to be detected by the ICT. This is the most probable reason why the sensitivity of the direct ICT was not high. We therefore spiked an equal volume of healthy control plasma, 2 h prior to ICT, to provide sufficient FXIII-A for detection by ICT (Suppl. Figure 1C, available online at www.thrombosis-online.com).

We applied this spiked ICT to plasma samples from 39 AH13-suspected patients. One patient was examined by spiked ICT alone, when we did not have enough assay reagents. As a result, 18 cases yielded a positive reaction in the spiked ICT, while 21 cases yielded a negative reaction. Three (AFD-9, AFD-10 and AFD-19) of the 18 positive samples assessed using spiked ICT were considered to be false-positives, as described above, while one (type B AH13 patients) of the 22 negative samples was considered to be a false-negative. Therefore, the specificity of the spiked ICT was 87% (20/23), while the sensitivity was 94% (15/16). The kappa coefficient of 0.79 was in the “good to fair” agreement range between the spiked ICT and the dot-blot analyses.

Performance of spiked ICT for detection of auto-Abs in patients with various types of AH13

The ICT spiked with healthy control plasma could detect auto-Abs in all patients with AH13 type Aa (from AH13-Aa-1 to -Aa-14) and one type Ab (AH13-Ab-3) (Figure 3B), but not in a type B case (AH13-B-3). We therefore applied the spiked ICT to various AH13 cases including types Aa, Ab, and B to evaluate its performance. All samples from AH13 type Aa cases, as well as two type Ab and one B cases (AH13-Ab-1 and -3, and AH13-B-1) yielded positive reactions, while the remaining three cases (AH13-Ab-2 and AH13-B-2 and 3) yielded negative reactions (Figure 3B).
 Modification of spiked ICT with FXIIIa for detection of AH13 type Ab

In order to improve the detection of AH13 type Ab, we spiked ICT with rFXIIIa pre-activated by thrombin because AH13 type Ab preferentially reacts with FXIIIa. As a result, we successfully detected auto-Abs in samples from all patients with type Ab (▶Figure 4A), but not in a healthy control plasma. This was also true for when the ICT spiked with healthy control plasma was performed after thrombin-treatment in the presence of calcium and the resultant serum was used (▶Figure 4B).

Correlation between ICT values and FXIII levels

Both direct and spiked ICTs (▶Figure 5) were significantly inversely correlated with FXIII activity levels but not with those of FXIII antigen in AH13 cases (Suppl. Figure 3, available online at www.thrombosis-online.com). Therefore, high ICT values may be implicated in pathogenic and pathologic conditions, i.e. a severe FXIII deficiency in AH13 patients.

Evaluation of direct and spiked ICTs by ROC curve analysis

We performed ROC curve analysis of our direct ICT by which patients with or without AH13 (24 healthy control, 23 AFD and 19 AH13) were diagnosed. As a result, the best cut off value of direct ICT was turned out to be 0.14 AU (Suppl. Figure 4, available online at www.thrombosis-online.com); the specificity and sensitivity of direct ICT were 89% (42/47) and 68% (13/19), respectively. The area under curve (AUC) was 0.74, which could be judged as moderately accurate (0.7<AUC<0.9) according to an arbitrary guideline (27). The specificity and sensitivity of direct ICT with a cut-off value of 0.18 AU (average + 2SD) were 94% (44/47) and 58% (11/19), respectively.

We also performed ROC curve analysis of spiked ICT by which patients with or without AH13 (24 healthy controls, 23 AFD and 20 AH13) were diagnosed. As a result, the optimal cut-off value for our spiked ICT was 0.23 AU; the specificity and sensitivity of spiked ICT were 98% (46/47) and 80% (16/20), respectively. The AUC was 0.91, which could be judged as highly accurate (0.9<AUC<1). The specificity and sensitivity of spiked ICT with a cut-off value of 0.18 AU (average + 2SD) were 91% (43/47) and 80% (16/20), respectively. These results indicated that the cut-off
values obtained by ROC curve analysis and by average + 2 SD made comparable performance in ICTs. We therefore kept cut-off values obtained by average + 2 SD.

**Discussion**

This is the first report of a rapid test for the detection of anti-FXIII-A auto-Abs, which cause a life-threatening bleeding disease termed AH13. In this study, we first characterised three in-house mAbs in detail to confirm the rational basis for their use in an important life-saving diagnostic method. We then developed an ICT to detect anti-FXIII-A auto-Abs and to differentiate AH13 cases from other HAF13D patients. The latter is essential for the proper treatment of AH13 because administration of immunosuppressive agents is indispensable for its Ab eradication therapy.

Among our three in-house mAbs against human FXIII-A, 1TH2–8C4C, had the highest affinity toward FXIII-A, and
Figure 5: The correlation between ICT values and FXIII levels. A) Values of direct (left) and spiked (right) ICTs are shown. The results are presented as box-and-whisker plots denoting the median, interquartile range, and the minimum and maximum data points. The values of both direct and spiked ICTs were significantly higher in AH13 cases than in the healthy control (HC) group (0.60 ± 0.61 vs 0.08 ± 0.05, P = 0.0073 and 0.78 ± 0.54 vs 0.08 ± 0.05, P < 0.0001, respectively) and AFD patients (vs 0.09 ± 0.07, P = 0.0085 and vs 0.11 ± 0.09, P < 0.0001, respectively). *; activated by thrombin in the presence of calcium. B) The correlation between direct and spiked ICTs are shown. There was a strong correlation between direct and spiked ICTs for both the AH13 (open circles) and AFD (closed circles) groups (R² = 0.62 and P = 0.0001; R² = 0.67 and P < 0.0001, respectively). C) Direct (left) and spiked (right) ICT values vs FXIII ammonia release (AR) activity (Act) levels are shown. For AH13 cases (open circles), a significant inverse correlation between both direct and spiked ICT values and FXIII-AR Act was observed (slope = –7.7, R² = 0.30, P = 0.022 and slope = –10.4, R² = 0.43, P = 0.0034, respectively).
The number of AH13 patients has recently been increasing in Japan. ICT values were significantly inversely correlated with FXIII activity levels, indicating an association between the quantity of anti-FXIII autoantibodies and the pathogenesis of AH13.

What is known about this topic?
- The number of AH13 patients has recently been increasing in Japan.
- The definitive diagnosis of AH13 can currently be established only through time-consuming and expensive laboratory tests, such as the immune/dot-blot assay and ELISA, to detect anti-FXIII autoantibodies.

What does this paper add?
- We successfully developed a rapid immunochromatographic test (ICT) to detect anti-FXIII-A autoantibodies and to differentiate AH13 cases from other haemorrhagic patients due to non-autoimmune FXIII deficiencies.
- ICT values were significantly inversely correlated with FXIII activity levels, indicating an association between the quantity of anti-FXIII autoantibodies and the pathogenesis of AH13.

Our ultimate goal is to make our ICT suitable as a POCT device (17, 19), the current methods may need to be optimized further. In addition, incorporating a positive control of anti-FXIII Abs into a test strip as a control line will improve the usefulness of the test because it will reduce the number of strips as well as reagents needed to perform the assay. Although the present ICT study was carried out using a bench top absorbance reader, it was possible to read results visually (by three independent examiners in our laboratory, data not shown).

In conclusion, we have developed a new, rapid, and easy-to-use ICT for anti-FXIII-A auto-Ab that diagnoses 90% of AH13 patients. This novel ICT method may be applicable to a POCT, and contribute to saving more AH13 patients’ lives.

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Author contributions
TO performed experiments and wrote the paper; SD, NK, and MY prepared in-house mAbs and developed ICT; SM assisted the experiments; AI created the research project and wrote the paper.

Conflicts of interest
None declared.
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