次年度は、AVWS症例の検体を用いて検証して、イムノクロマト法を試作する予定である。

F．研究発表

1. 論文発表:

Ichinose A, et al.(Kitajima I, 36人中18番目):
Inhibitors of Factor XIII/13 in older patients. Semin Thromb Hemost. 2014;40(6):704-11

G．知的財産権の出願・登録状況

1. 特許取得：なし
2. 実用新案登録：なし

厚生労働科学研究委託費（難治性疾患実用化研究事業）
委託業務成果報告（業務項目）

新凝固スクリーニングシステムに関わる技術開発

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研究要旨

21世紀の我が国は、世界でも有数の「少子超高齢社会」であり、「高齢者」の血栓症と出血症は言うに及ばず、「少子化」に拍車をかける「不育症」にも、抗リン脂質抗体症候群などの後天性凝固異常症が深く関わっている。そこで、長年にわたって実施されている、血小板数、PT, aPTTのみの凝固スクリーニング検査に、Fibrinogen、FDP（フィブリン／フィブリノゲン分解産物）と現行のD-dimerを加えて「一般凝固検査セット」とし、それらの検査では異常を検出できないFXIII/13活性、α_2-plasmin inhibitor活性、総plasminogen activator inhibitor-1濃度、von Willebrand因子活性などを「特殊凝固検査セット」として測定してそのデータを「統一特別検査」の結果として蓄積し、その有用性に関する前向き調査を開始した。平成26年度は、研究経費が2015年1月になるまで交付されなかったため研究実施期間が短かったにも拘らず、平成27年3月9日現在で合計19名の症例で統一特別検査と実験的精密検査を実施することができ、5例の自己免疫性出血病XIII/13、4例の後天性血友病A、１例の後天性von Willebrand症候群疑診の臨床検体をPOC検査の開発、検証のために確保したことも、今後の研究を推進するために大いに貢献するものと期待される。

A．研究目的

我々は、平成21年度から「後天性血友（出血）病XIII/13」、平成24年度から「出血性後天性凝固異常症」の班研究を実施し、平成25年度にこれらの疾患の「検査・診断のアルゴリズム」と「参照ガイド」を策定した。しかし、迅速かつ正確に抗凝固因子自己抗体やD-dimerを臨床現場で測定する検査キットがないため、各種の出血症、血栓症を「いつでもどこでも」確定診断する環境が整っていない。

そこで、１）標準的検査を用いて各種の後天性凝固異常症の症例を見逃さない「新スクリーニング検査セット」を開発すること、２）抗凝固因子抗体、FXIII/13活性、D-dimerを迅速、正確かつ安価に測定するイムノクロマトグラフィ（イムノクロマト；ICT）法などを開発するために、各種の後天性凝固異常症の臨床検体を収集するシステムの基盤を確立すること、３）その臨床検体の性状を精密に分析して確定診断への道標を開発することが、本研究の目的である。

B．研究方法

長年にわたり実施されている、血小板数、PT, aPTTのみの凝固スクリーニング検査を症例の主治医に実施して頂いて、予め凝固異常の検索を開始して頂く。主治医から研究協力者（採血キットを保管して検体収集にご協力頂いている研究者）を含む研究班にコンサルテーションして頂き、Fibrinogen、FDP（フィブリン／フィブリノゲン分解産物）とD-dimerを加えて「一般凝固検査セット」として測定し、それらでは異常を検出できないFXIII/13活性、α_2-plasmin inhibitor (α_2-PI)活性、総plasminogen activator inhibitor-1 (PAI-1)濃度、von Willebrand因子活性を「特殊凝固検査セット」として測定した。これらの検査により、本研究の対象である１）出血症の内、自己免疫性出血病XIII/13（Autoimmune Hemorrhaphilia XIII/13; AHXIII/13）、自己免疫性後天性von Willebrand症候群（Autoimmune acquired von Willebrand syndrome; AVWS）、後天性血友病A（Acquired Hemophilia A; AHA）などの自己免疫性出血症、２）血栓症の内、深部静脈血栓症と肺血栓塞栓症（肺静脈血栓塞栓症）、３）血栓と出血の両方を合併する血栓症・出血症の内、播種性血管内凝固症（Disseminated Intravascular Coagulation; DIC）と産婦人科的凝固異常症などを検索した。

（倫理面への配慮）

２６年度初頭に**山形大学医学部の倫理委員会**の承認を得た。臨床検体の提供に当たっては、各主治医が症例あるいはその家族から文書による同意を得た。

C．研究結果

1. 「一般凝固検査セット」と「特殊凝固検査セット」を合計すると21項目になり、迅速に検査して臨床データを症例と主治医に還元するために、全国規模の大手受託検査センターに「統一特別検査」として委託契約した。
2. この統一特別検査の実施を可能にするために「特別採血キット」を作製して、全国の研究協力者に２セットずつ配布し、症例に使用した後も不足しないように随時補充する体制を整えた。

3. 今年度の研究経費が2015年1月になるまで交付されなかったので研究実施期間が短かったにも拘らず、平成27年3月9日現在で合計19名の症例で統一特別検査と実験的精密検査を実施することができた。
4. これらの臨床症例は、5例のAHXIII/13、4例のAHA、1例のAVWS疑診、その他DICなど9例であり、これらの臨床検体を確保し、凍結保存した。

D．考察

原因不明の後天性凝固異常症を見逃すことなくスクリーニングするための21項目の「統一特別検査」を実施することにより、5例のAHXIII/13、4例のAHA、1例のAVWS疑診が研究班により精密検査されており、平成26年度においては「統一特別検査」自体の問題点は指摘されていない。勿論、thrombin activatable fibrinolysis inhibitorやtissue-plasminogen activator, urokinaseなどの線溶系タンパク質が含まれていないが、これらは国内最大の受託検査センターで実施されていないので、契約項目に入れることができなかった。従って、少なくとも現時点では、「標準的検査」の範囲で網羅的に出血性凝固異常症をスクリーニングするシステムを構築したと言える。

5例のAHXIII/13、4例のAHA、1例のAVWS疑診の臨床検体をPOC検査の開発と検証のために確保したことは、今後の研究を推進するために大いに貢献するものと期待される。勿論、これらの少数例のデータのみでは、統計学的に有意な所見を得ることはできないが、今後、複数年度のデータを蓄積することにより、医学的に意義のある結果をまとめることが可能になろう。

E．結論

対象疾患症例のスクリーニングのプロトコールを確立し、「統一特別検査」委託契約を結び、「特別採血キット」を作成して全国の研究協力者に配布したことにより、POCテスト開発のための「検体収集の基盤を構築」できたので、次年度からは全面展開する。

F．研究発表

1. 論文発表:
総括と共通であり、多数につき割愛した。

2. 学会発表
総括と共通であり、多数につき割愛した。

G．知的財産権の出願・登録状況

1. 特許取得：なし
2. 実用新案登録：なし

Ⅴ．学会等発表実績

様式第１９

学　会　等　発　表　実　績

委託業務題目「後天性凝固異常症のP.O.C.テストによる迅速診断システムの開発」
機関名　国立大学法人　山形大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
The current status of autoimmune hemorrhaphilia due to anti-FXIII/13 antibodies and its related diseases in Japan. 口演	Ichinose A	The 59th Annual Meeting of the Society of Thrombosis and Hemostasis Research, CCD Congress Center Düsseldorf (Düsseldorf, Germany)	February 24-27, 2015	国外
Management of autoimmune hemorrhaphilia XIII/13 (AH13) complicated by pulmonary thromboembolism. 口演	Ogawa Y, Yanagisawa K, Osaki T, Souri M, Ichinose A	The 59th Annual Meeting of the Society of Thrombosis and Hemostasis Research, CCD Congress Center Düsseldorf (Düsseldorf, Germany)	February 24-27, 2015	国外
Acquired Hemorrhaphilia Due to Anti-Factor XIII Autoantibodies. 口演	Ichinose A	Grand Rounds Lecture for the Division of Hematology/Oncology and the Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Northwestern University (Chicago, USA)	June 27, 2014	国外
Modification of the ISTH/SSC Bleeding Assessment Tool ver. 2010 and Its Field Test for Japanese Patients with Acquired Hemorrhaphilia due to Anti-F13 Autoantibodies. 口演	Ichinose A, Sugiura-Ogasawara M, Tosetto A, Rodeghiero F, James P.	The 60th Annual Scientific and Standardization Committee (SSC) Meeting of the International Society on Thrombosis and Haemostasis (ISTH), Subcommittee Sesstion (Factor XIII and Fibrinogen), Wisconsin Center (Milwaukee, USA)	June 23-26, 2014	国外
Recommendations for Criterion and Algorism of Laboratory Tests for Autoimmune Hemorrhaphilia Due to Anti-Factor XIII/13 Antibodies. 口演	Ichinose A, Kohler HP, Muszbek L, Philippou H. On behalf of the Factor XIII and Fibrinogen SSC Subcommittee of the ISTH.	The 60th Annual Scientific and Standardization Committee (SSC) Meeting of the International Society on Thrombosis and Haemostasis (ISTH), Subcommittee Sesstion (Factor XIII and Fibrinogen), Wisconsin Center (Milwaukee, USA)	June 23-26, 2014	国外

Two cases of acquired von wirebrand disease. ポスター発表	Matsuura Y, Takahashi Y, Yamaguchi M, Utsu Y, Masuda S, Aotsuka N, Wakita H, Matsushita T, Ichinose A	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
大量出血に伴う喪失により一過性の13因子欠乏症を合併した先天性結合組織疾患の症例. ポスター発表	山崎励至，野中博章，今西大介，一瀬白帝，宮崎泰司	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
抗血小板中止中に閉塞性動脈硬化症が憎悪し第13因子投与下でステント挿入術を行った後天性血友病13の1例. ポスター発表	平瀬伸尚， 惣宇利正善， 加藤愛子， 宇都宮勇人， 尾崎　司， 一瀬白帝	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
Knee hemarthrosis in the elderly with acquired factor 13 deficiency. ポスター発表	Nakashima H, Kawano K, Souri M, Ichinose A, Sakai A	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
肺血栓塞栓症を合併した抗第XIII/13因子自己抗体による後天性血友（出血）病の血栓止血学的マネージメント. ポスター発表	内藤千晶， 小川孔幸， 惣宇利正善， 尾崎　司， 三原正大， 清水啓明， 石埼卓馬， 柳澤邦雄， 三井健揮， 半田　寛， 野島美久， 一瀬白帝	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
Chronic intractable acquired hemorrhaphilia XIII/13 developed after surgery of bladder cancer. ポスター発表	Sugawara T, Sato Y, Ohwada C, Souri M, Osaki T, Fukasawa K, Komaru A, Tsujimura H, Maruyama S, Yamada S, Ise M, Kumagai K, Ichinose A	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
Rituximab投与にも拘らず脳内出血で死亡した重症自己免疫性出血病XIII/13の1症例. ポスター発表	Obayashi Y, Ohashi R, Murayama H, Kojima M, Souri M, Osaki T, Ichinose A	第76回日本血液学会学術集会，大阪国際会議場（大阪）	2014年10月31日～11月2日	国内
Characterization of plasma pro-transglutaminase, coagulation factor XIII, using its synthetic peptides. 口頭とポスター発表	尾崎　司， 惣宇利正善， 一瀬白帝	第87回日本生化学会大会，国立京都国際会館（京都）	2014年10月15-18日	国内

先天性凝固第XIII因子Bサブユニット欠損症例に生じた抗Bサブユニット同種抗体による活性阻害. 口頭とポスター発表	惣宇利正善, 尾崎　司, 和田秀穂, 一瀬白帝	第87回日本生化学会大会，国立京都国際会館（京都）	2014年10月15-18日	国内
自己免疫疾患を合併し、Rituximab投与とSteroid pulse療法で寛解に成功した後天性血友病13の１例. ポスター発表	三原正大, 小川孔幸, 惣宇利正善, 尾崎　司, 小林宣彦, 清水啓明, 入内島裕乃, 柳澤邦雄, 合田　史, 内海英貴, 野島美久, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）；	2014年5月29-31日	国内
複数の抗血小板薬投与が出血症状を誘発したと思われる後天性血友病（自己免疫性出血病）13の一例. ポスター発表	平瀬伸尚, 惣宇利正善, 加藤愛子, 宇都宮勇人, 有田好之, 生山祥一郎, 堀内孝彦, 尾崎　司, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）；	2014年5月29-31日	国内
２年間に２回免疫抑制療法に部分的反応を示したが再び増悪して治療中の難治性後天性血友病13の一例. ポスター発表	小島　稔, 惣宇利正善, 尾崎　司, 天木　惇, 沼田裕樹, 扇屋大輔, 坪井康介, 安藤　潔, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内
IL-6受容体抗体投与に伴う後天性第13因子（F13）欠乏症ではA subunit, B subunitが共に減少する. 口頭とポスター発表	惣宇利正善, 茂久田翔, 高杉　潔, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内
凝固XIII因子インヒビターの鋭敏な検出のための新規XIII因子活性測定法の開発. 口頭	惣宇利正善, 尾崎　司, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内
貴方の症例を救命するために：微修正ISTH/SSC BATからの日本語翻訳版出血スコア作成と実地試用. 口頭とポスター発表	一瀬白帝, 杉浦真弓, Rodeghiero F, Tosetto A, Paula J	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内
Plasminogen (PLG) 函館；代替性線溶機構の発動が推定された我が国２家系目の先天性PLG欠損症例. 口頭とポスター発表	尾崎　司, 惣宇利正善, 宋　勇錫, 泉　直宏, 太田啓宏, 小山創志, 周佐峻佑, 田中佑輔, 松尾草明, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内

第XIII因子濃縮製剤投与と免疫抑制療法実施にも拘らず出血死した後天性血友（出血）病13の劇症例. 口頭とポスター発表	金城恒道, 惣宇利正善, 梅崎徹也, 伊藤清信, 水野秀紀, 尾崎　司, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内
脳出血により死亡した、活性化第13因子に対する自己抗体陽性の後天性血友病（出血病）13の１例. 口頭	松浦真理, 惣宇利正善, 佐藤謙伍, 尾崎　司, 家子正裕, 中村順一, 鎌田啓佑, 岸野宏貴, 岩渕敬介, 廣海弘光, 渡部直己, 日下大隆, 小熊　豊, 一瀬白帝	第36回日本血栓止血学会学術集会，大阪国際交流センター（大阪）	2014年5月29-31日	国内
難治性後天性出血性疾患への挑戦. 口演	一瀬白帝	第75回山梨血液研究会，アピオ甲府（山梨）	2015年3月20日	国内
自己免疫性出血病XIII/13（Autoimmune Hemorrhaphilia XIII/13; AHXIII/13）の治療の現状について. 口演	一瀬白帝	第９回日本血栓止血学会学術標準化委員会(SSC)シンポジウムSSC Symposium 2015，野村コンファレンスプラザ日本橋（東京）	2015年2月28日	国内
自己免疫性出血病XIII/13（Autoimmune Hemorrhaphilia XIII/13; AHXIII/13）の診断基準について. 口演	一瀬白帝	第９回日本血栓止血学会学術標準化委員会(SSC)シンポジウムSSC Symposium 2015，野村コンファレンスプラザ日本橋（東京）	2015年2月28日	国内
出血性非自己免疫性FXIII/13欠乏症の診断と治療. 口演	一瀬白帝	第7回アンチトロンビンとプロテアーゼフォーラム，京王プラザホテル札幌（札幌）	2014年11月29日	国内
帰って来たプラスミノゲン. 口演	一瀬白帝	RIKEN SEMINER，理化学研究所（和光）	2014年10月20日	国内
出血性後天性凝固異常症への挑戦. 口演	一瀬白帝	第6回北海道後天性血友病診療研究会，センチュリーロイヤルホテル（札幌）	2014年 9月13日	国内
出血性後天性凝固異常症. 口演	一瀬白帝	第6回京都DICフォーラム，京都ホテルオークラ（京都）	2014年 9月12日	国内
帰って来たプラスミノゲン. 口演	一瀬白帝	第2回血栓竹田フォーラム，竹田市総合社会福祉センター（大分）	2014年 9月 6日	国内
出血と血栓から患者さんを救うための基礎知識. 口演	一瀬白帝	奥羽大学薬学部 教育研修・講演会，奥羽大学薬学部（福島）	2014年 7月10日	国内
自己免疫性出血病XIII/13の全て. 口演	一瀬白帝	第5回後天性第XIII(13)因子欠乏症研究会，シェラトン都ホテル大阪（大阪）	2014年 5月31日	国内

厚労委託研究の概要と東北地区後天性血友病の現状. 口演	一瀬白帝, 尾崎　司, 惣宇利正善	北日本後天性血友病診療ネットワーク学術集会，キャンパス・イノベーションセンター（東京）	2015年 3月 8日	国内
後天１３における抗F13自己抗体の特徴. 口演	惣宇利正善, 尾崎　司, 一瀬白帝	北日本後天性血友病診療ネットワーク学術集会，キャンパス・イノベーションセンター（東京）	2015年 3月 8日	国内
イムノクロマト法による後天１３症例の臨床現場迅速検査. 口演	尾崎　司, 惣宇利正善, 一瀬白帝	北日本後天性血友病診療ネットワーク学術集会，キャンパス・イノベーションセンター（東京）	2015年 3月 8日	国内
Characterization of plasma pro-transglutaminase, coagulation factor XIII, using its synthetic peptides. 口演	尾崎　司, 惣宇利正善, 一瀬白帝	第22回山形分子生物学セミナー，山形大学小白川キャンパス（山形）	2014年11月8日	国内
フィブリン架橋反応における抗XIII因子自己抗体の阻害作用機序. 口演	惣宇利正善, 尾崎　司, 一瀬白帝	第22回山形分子生物学セミナー，山形大学小白川キャンパス（山形）	2014年11月8日	国内
Characterization of plasma pro-transglutaminase, coagulation factor XIII, using its synthetic peptides. 口演	尾崎　司, 惣宇利正善, 一瀬白帝	第17回トランスグルタミナーゼ研究会学術集会，国立京都国際会館（京都）	2014年10月14日	国内
遺伝子組換えトロンボモジュリン製剤で改善した解離性大動脈瘤に合併した慢性播種性血管内凝固の一透析例. 口演	早川佳奈, 田村志宣, 早川隆洋, 大浦真紀, 谷口文崇, 木村りつ子, 中野好夫, 一瀬白帝, 藤本特三	日本内科学会第204回近畿地方会，大阪国際交流センター（大阪）	2014年6月14日	国内
凝固第XIII因子Bサブユニットとフィブリノゲンとの相互作用部位. 口演	惣宇利正善, 尾崎　司, 一瀬白帝	日本生化学会東北支部第80回例会・シンポジウム，アキタパークホテル（秋田）	2014年5月10日	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
Clinical features of 32 new Japanese cases with autoimmune hemorrha-philia due to anti-factor XIII antibodies.	Ichinose A, Souri M, Osaki T.	Haemophilia	2015	国外
Hemorrhagic Autoimmune Hemorrhaphilia Resulting from Autoantibody against the A Subunit of Factor XIII.	Uchida E, Watanabe K, Arai R, Yamamoto M, Souri M, Osaki T, Ichinose A, Miura O, Koyama T	Internal Medicine	2015	国外

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VI. 研究成果の刊行物・別刷

HAE	12677	WILEY	Dispatch: 6.3.15	CE: Saravanan
Journal Code	Manuscript No.		No. of pages: 6	PE: Sharanya

Haemophilia (2015), 1–6 DOI: 10.1111/hae.12677

ORIGINAL ARTICLE

Clinical features of 32 new Japanese cases with autoimmune haemorrha-philia[1] due to anti-factor XIII antibodies

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Summary. Autoimmune haemophilia-like disease (or haemorrha-philia) due to anti-factor XIII (FXIII; F13 to avoid confusion with FVIII or FXII) antibodies (termed AH13) is a severe bleeding disorder. Although AH13 is thought to be rare, 'the number of its diagnosed patients' has recently increased in Japan. However, its prevalence remains unknown. To improve understanding of this disease, we examined and diagnosed 32 'new' Japanese patients with AH13. The presence of antibodies against F13-A subunit and/or F13-B subunit was confirmed by using a dot blot test and enzyme-linked immunosorbent assays. Most of our patients had autoantibodies against the F13-A subunit (88%). A predominance of men (59%) was observed. The mean age and residual F13 activity of our AH13 cohort were 71.7 years and 10.5% of normal, respectively, and 53% of cases were idiopathic. Autoimmune disorders and malignancies were the leading underlying disease (both 16%). Intramuscular and subcutaneous bleeding were the leading symptoms (both 72%). Most of our patients were treated with F13 concentrates (72%) to arrest bleeding and with prednisolone (81%) to eradicate anti-F13 autoantibodies. Cyclophosphamide and rituximab (both 25%) were also administered. The mortality of AH13 was high (22%), and haemorrhage was the major cause of death (71%). Moreover, 13% of our AH13 patients were diagnosed after haemorrhagic death. Physicians/haematologists must raise the awareness of AH13 as a life-threatening disease. This report represents the only experience of a nationwide survey, and may contribute to a diagnosis on potentially overlooked non-Japanese AH13 patients in other countries in the world.

Keywords: autoimmune disease, chronic intractable disease, haemostatic therapy, immunosuppressive treatment, life-threatening bleeding disorder, nationwide survey

Introduction

Coagulation factor XIII (FXIII, or F13 is used to avoid confusion with FVIII and FXII for medical safety measure) is a fibrin-stabilizing factor and comprises a hetero-tetramer formed by two catalytic A subunits (F13-A) and two non-catalytic carrier B subunits (F13-B) [1–4]. Congenital F13 deficiency can cause lifelong severe bleeding and abnormal wound healing, and recurrent miscarriages in women. It is a very rare form of haemorrhagic disorder that occurs in only one in 3 million individuals [1], and approximately 600 cases have been identified worldwide [5]. By contrast, acquired F13 deficiency is much more common, frequently characterized by a non-autoimmune secondary decrease in F13 resulting from hyposynthesis and/or hyper-consumption of F13 due to primary diseases [3,4,6].

'Acquired haemophilia (AH) is an autoimmune disease characterized by the presence of autoantibodies directed against various clotting factors [7]'. The incidence of 'acquired haemophilia A (AHA) due to anti-FVIII(8) inhibitors' has been estimated at 1.5 cases per 1 million population per year [8].

In contrast, data on a few cases of 'autoimmune haemophilia-like disease (or hemorrha-philia) due to anti-F13 antibodies (termed AH13)' have been reported [3,9]. Only 19 cases with anti-F13 autoantibodies or acquired F13 inhibitors (excluding one patient with anti-fibrinogen antibodies and two patients with anti-F13 alloantibodies) were reported in the last century [10]. AH13 must be distinguished from non-autoimmune haemorrhagic acquired F13 deficiency (HAF13-def) [9] in that AH13 is more severe than HAF13def, and requires immunosuppressive therapy to eradicate autoantibodies, as well as F13 replacement therapy to stop bleeding. Nevertheless, even severe F13 deficiency in AH13 patients tends to be overlooked by clinicians, because thus far, there is no routine standard screening test to detect abnormal F13 activity.

The number of diagnosed AH13 cases recently seems to be on the increase in Japan [11] when compared to that of previously reported cases in the last century [12–19]. Accordingly, we investigated exclusively bleeding patients to clarify the actual status of AH13. As a result, we diagnosed 32 new Japanese patients with AH13 in the last 4 years. The 12 patients previously reported in ref. [11] were not included in duplicate in this cohort. Thus, this report represents the only as well as the largest experience of a nationwide survey documented, to date. The present manuscript aimed to characterize the presenting symptoms and outcomes of these AH13 patients to improve understanding of this disease.

Methods and materials

Recombinant F13-A (rF13-A) was a kind gift from Dr. P. Bishop of Zymogenetics (Seattle, WA). rF13-B was expressed using a baculovirus expression system and purified as previously described [20]. An anti-F13-A monoclonal antibody (mAb) was generously provided by Dr. G. Reed of Massachusetts General Hospital (Harvard Medical School, Boston, MA). A tetramethylbenzidine (TMB) peroxidase substrate kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

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. Written informed consent was obtained from all participants including AH13 cases of the present study.

All research procedures [inclusion criteria, blood collection, plasma F13 activity, enzyme-linked immunosorbent assay (ELISA) to detect anti-F13-B autoantibodies, dot blot assay for anti-F13-A or anti-F13-B autoantibodies, etc.] have been described previously [11,21,22], except for the ELISA to detect anti-F13-A autoantibodies, as described later. Briefly, since 2009 we officially embarked on a nationwide campaign against AH13, supported by the Japanese Ministry of Health, Labor, and Welfare. Flyers and a simple questionnaire on past cases of various types of acquired haemorrha-philia were sent to approximately 2000 university or public hospitals and haematologists. Patients who were bleeding actively due to unknown causes were recruited into the survey and were examined in detail. Inclusion criteria were [9,11]: healthy individuals who suddenly manifested severe bleeding symptoms without a family history of bleeding disorders, prolonged clotting times or platelet abnormalities, in which cases their physicians contacted members of the study group (Appendix).

Whole blood was collected into tubes containing a one-tenth volume of 3.2% sodium citrate. Plasma samples were quick-frozen and sent to Ƀ (SRL Ltd., Hachioji, Japan) for measurement of the plasma F13 activity with an ammonia release assay using a Berichrom FXIII kit (Siemens/Sysmex, Kobe, Japan), with a reference range of 70–140%.

Furthermore, suspected cases of AH13 were examined in Yamagata University for the presence of antibodies against F13-A and F13-B by using a dot blot test, as previously described [22]. For an anti-F13-A ELISA, 1 μL of plasma was incubated with 200 ng of rF13-A at 37°C for 2 h, and diluted 100-fold with a buffer. Ten microlitres of the diluted plasma was applied to a 96-well plate coated with anti-F13-A mAb and incubated. The plate was incubated with peroxidase-conjugated anti-human IgG. A 10-min assay was performed using TMB substrate. Cases with HAF13def secondary to other diseases, such as disseminated intravascular coagulation, were excluded easily because of the absence of anti-F13 antibodies.

For statistical analysis, values were expressed as mean ± SD or as median when specified. Comparisons between groups were performed using Kruskal–Wallis tests unless specified, or Fisher's exact tests (2 × 2 table) when appropriate, of SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC, USA). Differences were significant at a P-value of <0.05.

Results and discussion

Number of diagnosed AH13 cases in Japan

We diagnosed a total of 32 new AH13 cases during the last 4 years, from late 2010 through 2014 (Table 1). During the preceding 7 years, until early 2010, when we consulted physicians in charge of the AH13 cases, there was only 1 or 2 AH13 case per year [11]. However, the number of diagnosed AH13 cases has increased from late 2010, and 15 AH13 cases were diagnosed during 12 months (the Japanese business year between April 2013 and March 2014).

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[2]The members of Japanese Collaborative Research Group (JCRG) on AH13 are listed in Appendix.

Accepted after revision 13 February 2015

Table 1. Presenting characteristics of two Japanese patients with AH13.

	No.	(%)
Reported/diagnosed year		
2010	2	6
2011	3	9
2012	8	25
2013	12	38
2014	7	22
Total	32	100
Gender		
Female	13	41
Male	19	59
Total	32	100
Age category		
<40	0	0
<60	2	6
<80	23	72
≥80	7	22
Total	32	100
Sites of bleeding		
IM	23	72
SC	23	72
IC	4	13
IP	4	13
GI	3	9
Kidney/urinary	3	9
RetroP	2	6
PostOpe.	2	6
Comp	2	6
IJ	2	6
Spl Rap	1	3
Total	32	
Residual F13 activity		
0 to <5%	7	22
5 to <10%	15	47
10 to <20%	6	19
≥20%	4	13
Low	0	0
Total	32	100
Target of antibody		
IgG to F13-A	28	88
IgG to F13-B	5	16
Anti-F13	2	6
n.d.	0	0
Total	32	
Underlying disease		
None	17	53
Autoimmune disease	6*	19
Cancer	5	16
Diabetes mellitus	4	13
Hepatitis	3†	9
Schizophrenia	2	6
Syphilis	1	3
CML	1	3
Total	32	

n.d., not described; SC, subcutaneous; IM, intramuscular; GI, gastrointestinal; IC, intracranial; IJ, intrajoint; IP, intraperitoneal; PostOpe., operation site; RetroP, retroperitoneal; Comp., compartment syndrome; Spl Rap, spleen rupture; CML, chronic myelogenous leukaemia.
*One patient had two autoimmune diseases.
†Two patients had viral hepatitis.

There were only eight AH13 cases reported in the last century between 1986 and 1997 [12–19].

Because we began a nationwide survey in Japan in April 2009, the awareness of AH13 may have increased. Actually, leaflets, questionnaires and/or annual research report booklets on AH13 have been sent to approximately 2000 hospitals, twice every year since late 2009. In addition, Japan has become a rapidly ageing society, ranking first in the world, and therefore, the Japanese may be more susceptible to AH13, for some unknown reason related to compromised immune-tolerance. Finally, the number of diagnosed AH13 cases may have increased in the 21st century because its diagnosis has evolved over a long period since the last century. It is very likely that a concerted effort to identify these patients identified more patients.

Age and gender

The incidence of AH13 increased with age in our study sample (Table 1). The mean age was 71.7 ± 9.3 years (n = 32, median 70.5 years). There were more patients in the age group 60 to <80 years than other age groups (72%), when compared to patients in other age groups (P = 0.0005). In our cohort, AH13 did not occur in childhood or youth during the last 6-year period.

A predominance of male subjects was observed among our 32 AH13 patients (59% men and 41% women, Table 1), which is similar to the previous cohort [11].

Bleeding sites and symptoms

Bleeding symptoms among our 32 AH13 patients varied considerably (Table 1). Most of them bled in soft tissues, and intramuscular (72%) and subcutaneous (72%) bleeding were the leading sites of haemorrhage. In contrast, postsurgical bleeding was rarely observed (6%).

It is important to observe that intracranial bleeding occurred in 4 of our 32 AH13 cases (13%, Table 1). Furthermore, six patients manifested intraperitoneal or retroperitoneal bleeding (19%). One of our AH13 cases manifested spleen rapture spontaneously. These results indicate that AH13 is one of the most severe life-threatening haemorrhagic diseases.

It is important to note that the bleeding symptoms of AH13 are indistinguishable from those of 149 AHA cases in the United Kingdom [8]. Therefore, AH13 must be differentially diagnosed according to specific coagulation tests as proposed by our collaborative research group [11], but not depending upon haemorrhagic symptoms *per se*.

Residual F13 activity

The mean residual F13 activity was 10.5 ± 8.5% (n = 32; median 7%) among our AH13 patients (Table 1). More patients (15/32 = 47%) exhibited a residual F13 activity between 5% and <10% (arbitrary category) when compared to those with <5% (arbitrary category) of F13 activity (7/32 = 22%). Only one case of the former group had received F13 concentrates before blood collection. There was no clear relationship between the residual F13 activity and bleeding severity, because even patients having >10% of normal F13 activity (9/10, 90%) manifested spontaneous bleeding (Table 1), except for one patient who developed only postoperation bleeding.

These findings may merely reflect the fact that residual F13 activities in patients were measured in a commercial service laboratory using an ammonia release assay, which usually yields high background/blank values [23].

Major targets of F13 autoantibodies

Among our 32 AH13 patients, 88% developed anti-F13-A autoantibodies, whereas only five patients (16%) developed anti-F13-B autoantibodies (P < 0.0001; Table 1). Among them, two patients developed anti-F13-B autoantibodies alone, and the remaining three patients had both anti-F13-A and anti-F13-B autoantibodies.

These results suggest that AH13 patients with anti-F13-B autoantibodies may be overlooked because they usually manifest milder bleeding symptoms, as observed in congenital F13-B deficiency [3,4]. This is consistent with the fact that these two patients had relatively higher residual F13 activities, corresponding to 41%, and 13%, respectively. An exception was a Hungarian patient with severe disease who showed essentially no F13 activity (<1% of the normal values) [24]. Incidentally, F13 activity and F13-B antigen levels are normally not measured by general physicians.

Physicians should understand that immunological assays exhibit greater accuracy and sensitivity [25], and can detect both neutralizing and non-neutralizing antibodies, whereas functional assays detect immediately apparent inhibitors but do not detect non-neutralizing antibodies.

Underlying diseases

No underlying condition was found in 53% of our AH13 patients (Table 1), like AHA patients [8]. Of note, five patients (16%) had malignancies, two patients (6%) presented with viral hepatitis, and one patient presented with syphilis. Incidentally, no AH13 case was associated with pregnancy, in contrast to AHA cases in the United Kingdom [8]. Five AH13 patients had autoimmune disorders, which was also the leading associated disease (16%; Table 1). It is widely accepted that malignancies, pregnancy, and infectious and autoimmune diseases are related to compromised immune reaction to some extent.

Haemostatic treatment

Because of the presence of severe F13 deficiency due to anti-F13 autoantibodies, F13 concentrates were administered to most of our AH13 patients (72%, Table 2). Fortunately, F13 concentrates were approved by the public medical services tentatively in April 2013 and officially in September 2013 in Japan through our efforts.

Fresh frozen plasma (FFP) was less frequently infused into our patients (22%), while cryoprecipitate was not administered at all (cryoprecipitate is not commercially available in Japan). In addition, recombinant activated FVII (FVIIa) was not used in our AH13 cases [unpublished data of Japanese Collaborative Research Group (JCRG)], probably because AH13 patients have normal amounts of endogenous FVII, and consequently, do not need exogenous FVIIa. Unfortunately, there is neither an alternative nor bypassing agents for F13. This is a major difference from the haemostatic therapy for AHA [25,26].

Anti-fibrinolytic agents, such as tranexamic acid, were used in 44% of our AH13 patients (Table 2). Twelve of 16 physicians (75%) observed favourable effects of tranexamic acid on haemostasis in their patients (unpublished data of JCRG).

Table 2. Management and prognosis of AH13 cases.

	No.	(%)
Haemostatic treatment		
F13	23	72
RCC	6	19
FFP	7	22
Tr	14	44
Ad	3	9
None	3	9
n.d./unknown	1	3
Total	32	
Ab eradication		
PSL	26	81
CP	8	25
RTX	8	25
CS	4	13
Pulse	4	13
PE	2	6
IVIg	1	3
None	3	9
n.d./unknown	1	3
PSL only	9	28
Total	32	
Prognosis/outcome		
Recovered	5	16
Death	7	22
Under treatment	18	56
Monitoring	2	6
Total	32	100

n.d., not described; Ab, antibody; Ad, adrenalin; FFP, fresh frozen plasma; IVIg, intravenous immunoglobulin; RCC, red cell concentrates; Tr, tranexamic acid; CP, cyclophosphamide; CS, cyclosporine; PE, plasma exchange; PSL, predonisolone; Pulse, steroid pulse; RTX, retuximab.

Antibody eradication therapy

Most of our AH13 patients (81%) were initially treated with prednisolone (PSL; Table 2). Cyclophosphamide was administered as second-line therapy (25% of all cases). Steroid therapy alone may not be sufficient to suppress autoantibody production because only one out of our nine AH13 patients who were treated with PSL alone recovered (data not shown).

Rituximab, an anti-CD20 mAb, has been used in 25% AH13 patients (Table 2). Although RTX has been used in Japanese AH13 patients since 2010, it still has not been approved for AH13 treatment by the public medical insurance in Japan, and its off-label use is restricted for Japanese physicians who treat AH13 cases.

There was a scarcity in the use of high-dose intravenous immunoglobulin (3%) and the performance of plasma exchange (6%) in our AH13 patients. High-dose intravenous immunoglobulin is not recommended for AHA patients [26]. Of note, the effect of plasma exchange and immune-adsorption is transient because these procedures only remove or reduce anti-F13 autoantibodies in patents' plasma, but do not interrupt the synthesis of these autoantibodies.

The optimal immunosuppressive strategy has not been defined for AH13 yet. As more data become available by accumulating experience, the efficacy of these treatment regimens may justify recommendations of novel first-line therapies for AH13.

Outcome and prognosis

Unfortunately, only 16% of our patients primarily recovered from AH13, and 56% continued receiving treatment (Table 2). Moreover, seven patients (22%) died within a year. Five of the seven deaths (71%) were due to or related to haemorrhage (Table 3). Therefore, AH13 is still a life-threatening haemorrhagic disease. Moreover, among our 32 patients, four were 'dead on arrival of test samples'. These findings emphasize the need to raise the awareness of this disease.

Two AH13 patients have been on treatment for more than 2.5 years, even though they had successfully survived the life-threatening acute phase, which reinforces that AH13 should be considered a chronic intractable disorder.

The mean age of the seven non-survived patients (66.7 ± 12.0 years, Table 3) was lower than that of survived AH13 patients (73.0 ± 8.2 years, $n = 25$, $P = 0.12$; *ns*). No significant differences in the mean residual F13 activity were observed between non-survived patients (5.6 ± 1.6%) and survived cases (11.9 ± 10.8%, $P = 0.35$).

Effect of treatment on survival

Four of our seven (57%) patients who had received FFP died (vs. 3 out of 25 patients without FFP, 12%, $P = 0.026$ by Fisher's exact test). Two of seven non-survived patients (29%) did not receive F13 concentrates.

A third of our patients (3 out of 9 patients, 33%) who received only steroids died (vs. 7 deaths out of all our 32 patients, 22%, $P = 0.66$ by Fisher's exact test). By contrast, all four patients (100%, $P = 0.55$ by Fisher's exact test) who had undergone pulse steroid therapy survived.

Unfortunately, at least one of our 32 AH13 patients may have died of pneumonia during steroid therapy, and another patient has died from intestinal perforation during steroid tapering (Table 3). These results highlight the need to seriously consider the adverse effects of immunosuppressive therapies, as recommended for the treatment of AHA [8].

Conclusion

This manuscript represents the one and only as well as the largest and longest duration experience of a nationwide survey reported, to date. AH13 is a chronic intractable disease. Affected patients require long-term medical care. At the same time, AH13 is definitely a life-threatening disease. Unless treated promptly and aggressively [27], patient may die of haemorrhage either during the acute stage [28] or after a prolonged period. Finally, the prompt diagnosis and treatment of AH13 are essential to save patients' lives. We strongly recommend a long-term follow-up of AH13 patients, for several years following remission, similar to the strategy used for AHA [26]. If clinical symptoms indicate a relapse, testing and treatment should be initiated immediately.

Table 3. Cause and timing of death of AH13 cases.

Age	F13 activity (%)	Haemostatic treatment	Cause of death	Timing of death	
66	6	RCC, FFP	Bleeding (no F13 conc. dosing)	1 week before DOAS	7 weeks after admission
65	6	F13	Cerebral bleeding	1 week before DOAS	3 weeks after admission
60	7	F13, RCC, FFP, Tr	Haemorrhagic shock	5 weeks before DOAS	3 weeks after admission
79	7	Tr	Cerebral bleeding (no F13 dosing)	4 months before DOAS	2.5 months after admission
63	20*	F13, RCC, FFP, Tr, Ad	Suicide because of bleeding	4.5 months after diagnosis	6 months after admission
49	4	F13, FFP, Tr	Intestinal perforation	11 months after diagnosis	During steroid tapering
85	3	F13	Sepsis from infected skin ulcer	1 year after diagnosis	9 months after eradication off

Ad, adrenalin; F13 conc., F13 concentrates; FFP, fresh frozen plasma; RCC, red cell concentrates; Tr, tranexamic acid; DOAS, dead on arrival of test plasma samples to the first author's laboratory.
*After F13 conc. infusion.

Acknowledgements

This study was supported by research grants from the Japanese Ministry of Health, Labor, and Welfare for 5 years. We are deeply indebted to over 150 physicians for consulting their patients with severe acquired haemorrha-philia including AH13, Acquired von Willebrand syndrome, AHA, etc. We wish to thank Drs. Magari Y and Sugiyama D of Q-may Laboratory Inc. for their help in providing an immuno-chromatographic test kit to rapidly detect anti-F13-A autoantibodies and Ms. Shibue Y for her help in conducting this research project and in the preparation of this manuscript.

Authorship contribution:

AI planed and conducted the project, analysed data and wrote the paper. TO and MS carried out experimental examinations, analysed data and prepared the paper.

Disclosures

None declared.

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Appendix

Following were members of 'the Japanese Collaborative Research Group (JCRG) on AH13' for cooperation in conducting the project; Ichinose A, Souri M, Osaki T, Matsushita T, Urano T, Ieko M, Tamai Y, Ito T, Kawamae K, Yamamoto M, Madoiwa S, Kurosawa H, Ogawa Y, Yatomi Y, Hanafusa N, Kawasugi K, Ishida F, Kitajima I, Asakura H, Hosono N, Mouri H, Kobayashi T, Wada H, Miyata S, Kashiwagi H, Ikeda M, Yukawa M, Higasa S, Hato T, Okamoto K, Okamura T, Uchiba M, Hashiguchi T and Maruyama I.

Case Report Revised IM-4791-14-C R.1

Hemorrhagic Autoimmune Hemorrhaphilia Resulting from Autoantibody against the A Subunit of Factor XIII

Short running title; autoimmune hemorrhaphilia by FXIII inhibitor

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Abstract: 86 words
Text: 1622 words

Abstract

A 65-year-old woman was admitted with acute intramuscular hemorrhage of the left gluteus medius and piriformis muscles and associated anemia. Blood tests showed low plasma factor XIII (FXIII) antigen and activity. A cross-mixing test revealed a concave "inhibitor" pattern and anti-FXIII-A subunit antibody was detected. The patient was diagnosed with autoimmune hemorrhaphilia resulting from anti-FXIII antibody. The bleeding has not recurred since the initiation of treatment with oral immunosuppressive agents. Although hemorrhagic acquired FXIII deficiency is a rare disorder, prompt recognition of the underlying mechanism can save lives.

Key words: autoimmune hemorrhaphilia, anti-FXIII antibody, A subunit

Introduction

Factor XIII (FXIII) is a coagulation factor whose activated form contributes to hemostasis by promoting cross-links between fibrin monomers and stabilizing fibrin clots. FXIII is a pro-enzyme of two enzymatic A subunits (FXIII-A) and two non-catalytic B subunits (FXIII-B). Acquired factor XIII deficiency is a relatively common disorder, caused by decreased synthesis or increased consumption ofFXIII (1). Various conditions have been associated with acquired FXIII deficiency, including disseminated intravascular coagulation, major surgery, liver diseases, sepsis, Henoch–Schönlein purpura, and inflammatory bowel disorders (such as Crohn's disease and ulcerative colitis). The FXIII deficiency is mild and critical bleeding is rare. Conversely, autoimmune hemorrhaphilia resulting from anti-FXIII antibody (AHXIII) is a very rare, life-threatening bleeding disorder observed mostly in elderly patients. Only a total of 83 AHXIII cases have so far been diagnosed worldwide (2). AHXIII tends to become chronic and intractable. This disorder is not always recognized, and a late diagnosis may lead to patient death due to severe bleeding. We herein report here a case of AHXIII, where we promptly diagnosed, properly treated, and characterized the inhibitor in detail.

Case Report

A 65-year-old woman was admitted to our hospital with pain and a broad subcutaneous

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hemorrhage over the left thigh and buttocks. She had a history of acquired hypothyroidism due to Hashimoto's chronic thyroiditis since 20 years of age, type 2 diabetes, and chronic hepatitis C since 41 years of age; however, she had no prior history of bleeding tendency. The patient had received thyroid hormone replacement therapy. None of her family members had a history of bleeding. The patient had experienced pain in her right thigh when carrying heavy laundry up and down stairs, and pain began in her left thigh and buttocks 1 week later. On presentation at our hospital, the patient had a broad subcutaneous hemorrhage on the left thigh. Computed tomography revealed an intramuscular hemorrhage of the left gluteus medius and piriformis muscles (Fig. 1). Her hemoglobin level was 8.1 g/dL, which was down from 13.4 g/dL 2 months earlier. The patient was admitted to the Department of Vascular Surgery at our hospital and underwent embolotherapy of the superior and inferior gluteal arteries. The Department of Vascular Surgery consulted us regarding this hemorrhage. Her routine hemostatic tests were normal, but we found that she had a low plasma FXIII antigen concentration of 3% and FXIII activity of 8% (Table 1). FXIII antigen was determined with a latex agglutination assay (NS auto-FXIII; Kainos, Tokyo, Japan). FXIII activity was determined with a photometric ammonia release assay (Berichrom FXIII; Dade Behring, Marburg, Germany). No other abnormal hemostatic laboratory data were documented associated with the hemorrhage. A cross-mixing test showed a concave "inhibitor" pattern and markedly low plasma FXIII-A antigen levels. In contrast, the FXIII-B antigen levels were only 50% of the normal range (Fig. 2A, B). Anti-FXIII-A antibody was highly positive on an immuno-blot assay (Fig. 2C). This neutralizing anti-FXIII-A antibody should also induce the rapid clearance of FXIII-A and FXIII-B. A fibrin cross-linking study showed the lack of both γ–dimerization and α–polymerization, indicating that fibrin-stabilization was markedly impaired (Fig. 2D). We diagnosed the patient with acquired FXIII deficiency with FXIII-A inhibitor, AHXIII (1, 2).

Becauseactive hemorrhaging was not observed after embolotherapy, we did not administer FXIII and instead initiated oral prednisolone (PSL) therapy at 1 mg/kg (50 mg/day) (Fig. 3). Because a substantial hemorrhage did not recur, except for mild subcutaneous purpura on the knee, we decreased PSL by 5 mg every 2 weeks after 1 month of treatment. On day 73, the PSL dose was 30 mg/day. The plasma FXIII activity and antigen levels mildly improved to approximately 12–22% and 20–23%, respectively, after PSL was initiated, indicating that the inhibitor activity still remained. To treat this

ongoing problem, we added cyclophosphamide (CPA) at 50 mg/day to PSL (30 mg/day) beginning on day 94. Since that time, no hemorrhage has been documented, and the residual FXIII activity in the 1:1 cross-mixing test increased from 7% to 40%. Due to the patient's worsening diabetes and osteoporotic vertebral compression fractures, and because of PSL's insufficient inhibitor-eradication effect, we gradually decreased the PSL dosage and continued CPA at 50 mg/day. Even after the addition of CPA, the plasma FXIII activity and antigen levels did not increase to more than 26% and 22%, respectively (Fig. 3). Because health insurance restrictions prevented us from administering anti-CD20 rituximab, which is not approved for AHXIII in Japan or at our university hospital, we replaced CPA with cyclosporin A (CyA) at 4 mg/kg (200 mg/day) along with 7 mg/day of PSL beginning on day 204. The FXIII activity and antigen level remained low with the addition of CyA, but the residual FXIII activity increased (Fig. 3). In fact, total anti-FXIII-A IgG decreased markedly to less than 10% of the initial level at onset (data not shown). Furthermore, bleeding has not recurred. We gradually decreased the CyA dose and the patient is currently being maintained on 100 mg/day of CyA and 5 mg/day of PSL.

Discussion

AHXIII occurs most commonly in the elderly, with a mean patient age of 70.4 years in Japan (2). Our patient was a 65-year-old woman and presented with typical spontaneous intramuscular bleeding. FXIII deficiency in children is generally congenital, but a case of a 9-year-old girl with AHXIII has been reported (3). Approximately half of all AHXIII cases are idiopathic and aging may be one of the most important factors for AHXIII, probably due to the loss of "self-immune tolerance" (2). The remaining half of the cases has some underlying disorders, such as autoimmune diseases and malignant tumors, or may be due to pharmaceutical agents, such as isoniazid, phenytoin, penicillin, and ciprofloxacin (2, 4, 5). Severe liver disease may also cause FXIII deficiency because of decreased synthesis of FXIII-B in the liver; however, in the present case, chronic hepatitis C was well controlled and the patient had an inhibitor against FXIII-A. The present patient also had diabetes mellitus and autoimmune hypothyroidism, both of which were well controlled. The relationship between acquired von Willebrand syndrome and hypothyroidism is well documented (6), and AHXIII has been reported in a patient with congenital hypothyroidism (7), however, there are no current reports of AHXIII in patients with acquired

hypothyroidism. Our patient had a history of Hashimoto's chronic thyroiditis for more than 40 years, and the onset of AHXIII was not found to be associated with the deterioration of thyroid function. While the loss of "self-immune tolerance" may be common between Hashimoto's thyroiditis and AHXIII, a direct relationship between those disorders has not yet been shown.

FXIII concentrate, fresh frozen plasma, and cryoprecipitate have been used to control bleeding in patients with AHXIII. To eradicate coagulation inhibitors, corticosteroids, CPA, CyA, and rituximab are typically administered, according to the acquiredhemophilia guidelines (2, 8). In the present case, we initially treated the patient with PSL, which was mildly effective. Neither CPA nor CyA were effective. The efficacy of rituximab for acquired inhibitors of coagulation factors, including AHXIII, has been reported in several studies (9, 10). However, we were not able to use rituximab in this case because its use for this disorder is not currently accepted at our hospital. In other previously studies in which steroids, CPA, and CyA were ineffective (11, 12), alternative treatments, such as plasmapheresis, immunoglobulin, and rituximab, were used, resulting in increased FXIII activity. In the present case, active bleeding has not recurred since the patient began PSL treatment, which represents clinical remission. Thus, a mild increase in the FXIII antigen and activity and residual FXIII activity may be sufficient for hemostasis. However, an increased risk of bleeding persists because the patient's FXIII activity remains low, which means that laboratory or immunological remission (2) has not yet been obtained. We must closely follow the patient for a long period, and if bleeding recurs, we plan to administer steroid pulse therapy with methyl-PSL with or without FXIII concentrate. Because FXIII is a rather polymorphic protein (13), and because a FXIII concentrate "Fibrogammin®" made from Caucasian plasma is now available in Japan, a booster effect of the FXIII concentrate on the production of anti-FXIII antibody is anticipated. Therefore, we have refrained from infusing the FXIII concentrate in the present case where the bleeding was not severe. The "watch and wait" strategy, along with the maintenance of mild immunosuppressive therapies, seems to be clinically relevant for older patients with risks of serious adverse events, such as in the present case.

Hemorrhagic cases of acquired inhibitors of coagulation factors, such as acquired hemophilia A, have been on the rise in Japan, which has become the leading "super-aging" society. In FXIII deficiency, the results of routine coagulation tests (such as prothrombin time and activated partial thromboplastin

time) are normal. As a result, some patients with FXIII deficiency may be misdiagnosed. Moreover, AHXIII patients may bleed profusely. While intramuscular and subcutaneous bleeding are common, most fatal bleeding is intracranial (2, 5). In some patients, this disorder is diagnosed after autopsy in massive and fatal bleeding cases (14). To decrease the number of bleeding deaths among patients with AHXIII, we have to carefully examine hemostatic tests, including FXIII antigen and activity, to diagnose AHXIII and begin treatment as early as possible. The full characterization of the inhibitor as presented in this report may also be essential in initiating the most appropriate anti-hemorrhagic therapy and to remove related conditions to eradicate the inhibitor and to improve the patient's outcome (5). More nationwide and worldwide surveying is necessary to establish the treatment strategies.

Acknowledgment

We greatly thank Dr. Soutaro Katsui (Department of Vascular Surgery, Tokyo Medical and Dental University) for the referral of this patient to our department.

The authors declare that they have no conflicts of interest (COI).

This report was presented in part at the 604th Kanto regional meeting of the Japanese Society of Internal Medicine held in Tokyo, Japan where it won the Incentive Award. The analyses of FXIII inhibitor were supported by a research grant from the Japanese Ministry of Health, Labour and Welfare.

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Table 1. Laboratory data on admission. Hemostatic parameters included reduced plasma levels of FXIII antigen and activity.

Figure legends

Figure 1. Intramuscular hemorrhage of the left gluteus medius and piriformis muscles confirmed by computed tomography on admission.

Figure 2. (A) Cross-mixing test. A five-step dilution cross-mixing test with an amine incorporation assay was performed using the patient's plasma at ratios of 0:1, 1:3, 1:1, 3:1, and 1:0 with normal plasma. The mixed samples showed a concave "inhibitor" pattern. **(B) Enzyme-linked immunosorbent assay (ELISA) of FXIII antigen in the patient's plasma.** The plasma FXIII-A levels were markedly low. In contrast, the FXIII-B levels were reduced by no more than one-half. **(C) Dot blot analysis of FXIII-reactive immunoglobulins.** A dot blot analysis was performed using recombinant FXIII-A (rFXIII-A), recombinant FXIII-B (rFXIII-B), and their complexes (rFXIII(A_2B_2)) at the indicated amounts, shown as ng of antigen. The positive controls were plasma from patients with previously confirmed AHXIII. The results showed that anti-FXIII-A antibody was positive, but anti-FXIII-B antibody was negative, indicated that the patient suffered from acquired factor XIII deficiency with FXIII-A inhibitor. **(D) Fibrin cross-linking study.** The fibrin cross-linking study was performed by adding 1 unit/mL thrombin and 5 mM $CaCl_2$ to the patient's plasma and to normal control plasma. Clots were recovered at the indicated

time intervals and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The results indicated the lack of both γ–dimerization and α–polymerization.

Figure 3. Clinical course after hospitalization. The plasma FXIII antigen levels were elevated mildly after starting prednisolone (PSL), but remained near 20%. The FXIII activity levels were elevated only slightly at approximately 12–22% with PSL, cyclophosphamide (CPA), or cyclosporine A (CyA) as immunosuppressive treatments. The residual FXIII activity is the FXIII activity in a 1:1 cross-mixing test incubated for 2 hours at 37°C. A mild increase in the residual activity was observed with immunosuppressivetreatment. A Japanese version of the ISTH/SSC Bleeding Assessment Tool was utilized to objectively evaluate the patient's bleeding symptoms (2). The bleeding score was 7 at admission to our hospital and promptly decreased following the administration of immunosuppressive medication.

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 9.3×10^{-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

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Received: September 8, 2014
Accepted after major revision: January 10, 2015
Epub ahead of print: March 5, 2015
http://dx.doi.org/10.1160/TH14-09-0745
Thromb Haemost 2015; 113: ■■■

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 γ-glutamyl-ε-lysine residues in fibrin and the α_2-plasmin inhibitor as well as fibrin monomers to form stable fibrin clots with increased resistance to mechanical stresses and fibrinolysis (1–4).

Acquired FXIII deficiency (AFD) 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 haemorrha-philia; termed AH13 in this manuscript), which manifests more severe bleeding symptoms than non-autoimmune haemorrhagic AFD (HAFD) (5, 6).

Acquired FXIII inhibitors, especially auto-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 transaminase 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 kenowlege. 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 HAFD 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)-NH_2 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 $CaCl_2$, 0.2 % N,N-dimethylcasein, 2 mM monodansylcadaverine and 1 U bovine thrombin as previously described (8, 10, 21).

The effect of mAbs on the formation of the FXIII-A_2B_2 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-A_2B_2 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 $CaCl_2$. A western blot of the digested product was performed