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Novel heterozygous dysfibrinogenemia, Sumida (AαC472S), showed markedly impaired lateral aggregation of protofibrils and mildly lower functional fibrinogen levels

Minami Ikeda [a,b], Shinpei Arai [b,c], Saki Mukai [b, d], Yuka Takezawa [b], Fumiko Terasawa [a,d], Nobuo Okumura [a,d]

[a] Department of Clinical Laboratory Investigation, Graduate School of Medicine, Shinshu University, Matsumoto, Japan
[b] Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan
[c] Department of Laboratory Medicine, Graduate School of Medicine, Shinshu University, Matsumoto, Japan
[d] Department of Health and Medical Sciences, Graduate School of Medicine, Shinshu University, Matsumoto, Japan

Corresponding author: Nobuo Okumura
Laboratory of Clinical Chemistry and Immunology,
Department of Biomedical Laboratory Sciences,
School of Health Sciences, Shinshu University,
3-1-1 Asahi, Matsumoto, 390-8621 Japan
Tel.: 81-263-37-2392
Fax: 81-263 37-2370
Email: nobuoku@shinshu-u.ac.jp

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Abstract

Introduction: We encountered a 6-year-old girl with systemic lupus erythematosus. Although no bleeding or thrombotic tendency was detected, routine coagulation screening tests revealed slightly lower plasma fibrinogen levels, as determined by functional and antigenic measurements (functional/antigenic ratio = 0.857), suggesting hypodysfibrinogenemia. ***Materials and methods:*** DNA sequence and functional analyses were performed on purified plasma fibrinogen, and recombinant variant fibrinogen was synthesized in Chinese hamster ovary cells based on the results obtained. ***Results:*** DNA sequencing revealed a heterozygous AαC472S substitution (mature protein residue number) in the αC-domain. AαC472S fibrinogen indicated the presence of additional disulfide-bonded molecules, and markedly impaired lateral aggregation of protofibrils in spite of slightly lower functional plasma fibrinogen levels. Scanning electron microscopic observations showed a thin fiber fibrin clot, and t-PA and plasminogen-mediated clot lysis was similar to that of a normal control. Recombinant variant fibrinogen-producing cells demonstrated that destruction of the Aα442C-472C disulfide bond did not prevent the synthesis or secretion of fibrinogen, whereas the variant Aα chain of the secreted protein was degraded faster than that of the normal control. ***Conclusion:*** Our results suggest that AαC472S fibrinogen may cause

dysfibrinogenemia, but not hypofibrinogenemia. The destruction and steric hindrance of the αC-domain of variant fibrinogen led to the impaired lateral aggregation of protofibrils and t-PA and plasminogen-mediated fibrinolysis, as well as several previously reported variants located in the αC-domain, and demonstrated the presence of disulfide-bonded molecules.

Key Words: fibrinogen, dysfibrinogenemia, lateral aggregation of protofibrils, fibrin fiber, clot lysis

Abbreviations: APTT; activated partial thromboplastin time, CHO; Chinese hamster ovary, ELISA; enzyme-linked immunosorbent assay, HBS; N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], pH 7.4, 0.12 M NaCl, HEPES; N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid], HMW; high molecular weight, LMW; low molecular weight, LMW’; low molecular weight prime, PAGE; polyacrylamide gel electrophoresis, PCR; Polymerase chain reaction, PT; Prothrombin time, SDS; sodium dodecyl sulfates, SEM; Scanning electron microscopy, t-PA; tissue type-plasminogen activator.

Introduction

Fibrinogen is a 340 kDa plasma glycoprotein composed of two sets of three different polypeptide chains (Aα, Bβ, and γ) [1, 2], and physiologically functions in blood coagulation, fibrinolysis, wound healing, and angiogenesis [3]. Each chain is coded by the *FGA*, *FGB*, *FGG* genes, respectively, and then synthesized, assembled into a six-chain molecule in hepatocytes, which is expressed as $(A\alpha, B\beta, \gamma)_2$, and secreted into the blood. The six chains are arranged into three globular regions (a central E region and two distal D regions) connected by linear segments called coiled-coil connectors. The E region contains the N-termini of all chains [3].

During blood coagulation, thrombin cleaves four short peptides from fibrinogen to form fibrin monomers. In brief, two sets of fibrinopeptide A and fibrinopeptide B are released from the N-termini of the Aα and Bβ chains, respectively, which exposes the knobs 'A' and knobs 'B'. The fibrin monomers spontaneously polymerize in a two-step fashion [3]. In the first step, the so-called knob 'A', hole 'a', and D-D interactions form half-staggered, double-stranded protofibrils and the second step of polymerization, termed lateral aggregation, occurs. The release of fibrinopeptide B promotes lateral aggregation [4]. The final product is an insoluble fibrin network consisting of multi-stranded, branched fibers [3].

Genetic mutations in fibrinogen chain genes have been implicated in afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia, as listed on the GEHT homepage [5] (updated on 26/01/2012). As many as 307 families with dysfunctional fibrinogens have been analyzed genetically and/or structurally. Most of these variants are present in either the Aα chain (46 species, 170 families) or γ chain (49 species, 97 families), while variants of the Bβ chain have been detected in 40 families (24 species). Three clinical manifestations; a tendency towards bleeding (26.1 %), thrombosis (18.6 %), and renal amyloidosis (7.2 %), are also associated with dysfibrinogenemia, whereas 48.2 % of carriers are asymptomatic. The αC-domains of the fibrinogen molecule (Aα chain amino acid sequence 392-610) are folded into a globular structure, interact with the central region of the molecule, and play an important role in enhancing lateral aggregation [6, 7]. Furthermore, several variants have been identified in this domain, namely truncated or albumin-binding forms or pathogenic forms of familial renal amyloidosis [5].

We recently found a novel heterozygous dysfunctional fibrinogen, in which Ser was substituted for Aα472Cys in the αC-domain, analyzed its molecular characterization, fibrin polymerization, fibrin clot and fiber structure, and clot lysis by tissue-type plasminogen activator (t-PA)-mediated plasminogen activation, and compared these

with those of other fibrinogen variants located in the αC-domain, which are present as albumin-binding variant fibrinogens.

Materials and methods

This study was approved by the Ethics Review Board of Shinshu University School of Medicine. After informed consent had been obtained from the parents, blood samples were collected for biochemical and genetical analyses.

Patient

The proposita was a 6-year-old girl with systemic lupus erythematosus. Since she was positive for serum anti-cardiolipin-$\beta_2$glycoprotein I antibodies, she had taken warfarin to prevent the thrombosis associated with anti-phospholipid syndrome. Before being diagnosed, her plasma fibrinogen concentration was continuously lower than the lower-level of reference intervals without bleeding or a thrombotic tendency. Her mother had a similar plasma fibrinogen concentration to the proposita, but had no history of bleeding or a thrombotic tendency, and her father's plasma fibrinogen concentration was normal.

Coagulation screening

Nine volumes of blood were collected from the intermediate cephalic vein into plastic tubes containing one volume of 3.2 % trisodium citrate. Plasma was separated by centrifugation at 1500 x *g* for 10 minutes at 4 °C. The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration determined by the thrombin time method were measured with the automated analyzer, Coagrex-800 (Sekisui Medical Co., Tokyo, Japan). The immunological fibrinogen concentration was determined using anti-fibrinogen antibody-coated latex particles (LSI Medience Co., Tokyo, Japan) [8].

Polymerase chain reaction (PCR) and DNA sequence analysis

Genomic DNA was extracted from white blood cells using a DNA Extraction WB Kit (Wako Pure Chemical Ltd, Osaka, Japan), according to the manufacturer's instructions. To analyze all exons and exon–intron boundaries of fibrinogen genes, long-range PCR for *FGA*, *FGB* and *FGG*, and direct DNA sequencing for their purified PCR products were performed as described elsewhere [9].

Purification of plasma fibrinogen

Fibrinogen was purified from citrated plasma obtained from the proposita, her mother, and a normal control subject by immunoaffinity chromatography using an

IF-1monoclonal antibody (LSI Medience Co), and concentrations were determined as described elsewhere [9]. The purity of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reduced conditions (10% polyacrylamide gel). The characterization of the proteins was determined by SDS-PAGE under non-reduced conditions (5% polyacrylamide gel) followed by an immunoblot analysis with a rabbit anti human fibrinogen antibody (Dako, Carpinteria, CA, USA) and horse radish peroxidase-conjugated goat anti rabbit IgG antibody (Medical and Biological Laboratories Inc., Nagoya, Japan), or a rabbit anti-human albumin antibody (Dako) with the reacting species being visualized with the aid of an alkaline phosphatase-conjugated goat anti rabbit IgG antibody (EY Laboratories Inc., San Mateo, CA, USA) as described elsewhere [10].

Preparation of mutant expression vectors and recombinant variant fibrinogens

Recombinant variant fibrinogens were prepared as described previously [11]. Briefly, the variant fibrinogen Aα chain expression vector, pMLP-Aα472S or -Aα442S, was altered from pMLP-Aα at codon 472 TGT (Cys) to TCT (Ser) or 442 TGC (Cys) to TCC (Ser) by oligonucleotide-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following primer pairs (the altered base is underlined); 5'-GAT GGT TCT GAC T<u>C</u>T CCC GAG

GCA ATG-3’ (sense) and 5’-CAT TGC CTC GGG AGA GTC AGA ACC ATC-3’ (antisense) for Aα472S, 5’-CCA CGC GTC GTT CAT CCT CTA AAA CCG TTA C-3’ (sense) and 5’-GTA ACG GTT TTA GAG GAT GAA CGA CGC GTG G-3’ (antisense) for Aα442S [11]. The resultant expression vector for Aα472S or Aα442S was co-transfected with the histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells that expressed normal human fibrinogen Bβ and γ chains (Bβγ-CHO cells) as described elsewhere [11]. Ten colonies from each variant of fibrinogen-synthesizing CHO cells (Aα472S- or Aα442S-CHO cells) were selected at random and expanded to examine the synthesis of fibrinogen. Fibrinogen concentrations in culture media or cell lysates were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [11]. Both recombinant fibrinogens, Aα472S and Aα442S, were purified from harvested serum-free medium by immunoaffinity chromatography and concentrations were determined as described elsewhere [9].

Thrombin-catalyzed fibrin polymerization and thrombin clotting time

Polymerization was followed by measurements of changes in turbidity at 350 nm at ambient temperatures using the UVmini-1240 spectrophotometer (Shimadzu, Tokyo, Japan). Reactions were performed as described elsewhere [9] using 20 mM

N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl (HBS buffer) and human α-thrombin (Enzyme Research Laboratories, South Bend, MA, USA). Three parameters: lag time, the maximum slope, and absorbance changes for 30 min (final OD), were obtained from the turbidity curves, as previously described [12]. Thrombin clotting time was also measured in duplicate as described elsewhere [8].

Scanning electron microscopy (SEM)

To make fibrin clot, thrombin (10 μL at 0.5 U/mL) was added to fibrinogen solution (40 μL at 0.4 mg /mL). The SEM preparation was performed as described previously [9]. Images were viewed on a JSM-6000F (Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan) and taken at 3,000x or 20,000x with a 5-kV accelerating voltage. Fiber diameters were measured using a vernier clipper on a 300% enlargement from a photograph of a 20,000x observation.

Clot lysis assay

The clot lysis assay was performed with plasmin or a mixture of the two-chain tissue type-plasminogen activator (t-PA) and plasminogen, as described previously [10]. Briefly, 100 μl of purified fibrinogen was polymerized in a micro-quartz cell with 10

U/mL of human α-thrombin in polymerization buffer containing 1.0 mM $CaCl_2$ and was then incubated for 2h at 37 °C. The final concentrations of fibrinogen and thrombin were 0.45 mg/mL and 1.0 U/mL, respectively. After the completion of polymerization, 100 μl of plasmin (Chromogenix AB, Molngal, Sweden) (final concentration: plasmin:0.25 U/mL) or a mixture of t-PA (Genetech, South San Francisco,CA) and plasminogen (Roche Diagnostics GmbH, Mannheim, Germany) (final concentration: t-PA:1000 U/mL, and plasminogen: 1.0 U/mL) (t-PA/plasminogen mixture) was overlaid onto the clot, and clot lysis was monitored as a decrease in turbidity at 350 nm. Three parameters: lag time [the time until maximum absorbance (as 100 %) decreased to 98 %], the maximum lysis rate (Lmax), and the 50 % lysis time (the period until maximum absorbance decreased to 50%), were obtained from the turbidity curves. All reactions were performed in duplicate.

Statistical analysis

The significance of differences between wild-type and variant fibrinogen was determined using a one-way ANOVA (analysis of variance) and Tukey-Kramer tests. A difference was considered significant when $p < 0.01$.

Results

Coagulation screening tests and DNA sequence analysis

The results of the screening tests revealed that the proposita's plasma fibrinogen concentration determined by the thrombin time method and immunological method were low; 1.14 g/L and 1.33 g/L (normal range: 1.80 to 3.50 g/L), respectively. PT was 19.3 sec (normal range: 10.0 to 13.0 sec) and APTT was 27.6 sec (normal range: 23.0 to 28.0 sec). The prolongation of PT was attributed to the oral administration of warfarin to prevent thrombosis. The mother's plasma fibrinogen concentration determined by the thrombin time method was 1.41 g/L, which was less than the normal range, but was 1.90 g/L and within normal ranges when determined by the immunological method. PT and APTT were 11.4 sec and 31.0 sec, respectively.

A DNA sequence analysis for proposita and her mother revealed a novel heterozygous mutation of G>C *FGA* at exon 5 (at c.1472G>C; NCBI NM_021871), resulting in the substitution of Cys (TGT) for Ser (TCT) at the Aα472 residue (mature protein) or Aα491 (native protein) (AαC472S). We designated this dysfibrinogenemia as Sumida, according to the place of residence of the proposita.

Characterization of purified plasma fibrinogen

SDS-PAGE under reducing conditions of purified normal plasma fibrinogen, fibrinogen Sumida, and Sumida's mother showed the typical pattern of 3 bands corresponding to the Aα, Bβ, and γ chains (Figure 1A). Although Coomassie-stained gels for normal fibrinogen under non-reducing conditions indicated the three band pattern consistent with high-molecular-weight (HMW)-fibrinogen (340 kDa), low-molecular-weight (LMW)-fibrinogen (305 kDa), and faint low-molecular-weight-prime LMW' (270 kDa)-fibrinogen (Figure 1B, Lane 1) [13], three additional bands were present for fibrinogen Sumida and Sumida's mother and all were larger than HMW-fibrinogen (Figure 1B, Lanes 2 and 3; numbered as Band-1 to -3, respectively).

To further analyze these additional bands, fibrinogen was run on 5% SDS-PAGE followed by an immunoblot analysis using with a rabbit anti-human fibrinogen antibody (Figure 1C) or rabbit anti-human albumin antibody (Figure 1D). Band-1 to band-3 were reacted with the anti-fibrinogen antibody and a faint fourth band between band-3 and HMW-fibrinogen could be visualized, which was indicated as band-4 (Figure 1C). Band-2 and band-4 were also reacted with the anti-albumin antibody (Figure 1D). We speculated that band-1 was the dimer of variant Aα472S-fibrinogen while both band-2 and band-4 were variant fibrinogen-albumin complexes.

Thrombin-catalyzed fibrin polymerization and thrombin clotting time

Thrombin-catalyzed fibrin polymerization was performed as described in the Materials and methods. Under conditions with 0.05 U/mL thrombin, polymerization for fibrinogen derived from Sumida and Sumida's mother were markedly reduced; the maximum slopes (3.5 ± 0.2 and 3.5 ± 0.5 Abs x 10^{-4}/sec, respectively) were 3.1-fold slower than the normal control (10.9 ± 1.2) and final OD (0.157 ± 0.005 and 0.137 ± 0.001) were 2.2- and 2.6-fold lower than the normal control (0.346 ± 0.011) (Figure 2A). However, the lag periods (180.0 ± 0.0 for Sumida and 216.0 ± 17.0 sec for Sumida's mother) were similar to that of the normal control (189.0 ± 12.7 sec). These results were markedly different from the slightly lower functional fibrinogen levels determined by the procedure based on the thrombin time method.

Thrombin concentration was marked difference in reaction conditions between the thrombin time method and the thrombin-catalyzed fibrin polymerization, namely 33 U/mL and 0.05 U/mL, respectively. Therefore, we performed thrombin-catalyzed fibrin polymerization under the condition of 0.5 U/mL thrombin. In general, under the condition of 0.5 U/mL thrombin, the parameters of the maximum slope and final OD of fibrin polymerization curve are steeper and lower, respectively, than those obtained under the condition of 0.05 U/mL thrombin. These changes in plasma fibrinogen

derived from Sumida and Sumida's mother were not significantly different from the degree of change obtained with normal fibrinogen. The maximum slopes (Sumida: 23.0 ± 1.4 and Sumida's mother: 16.8 ± 6.0 Abs x 10^{-4}/sec, respectively) were 2.7- and 3.7-fold slower than the normal control (62.0 ± 2.8), and final OD (0.106 ± 0.010 and 0.089± 0.030) were 2.0- and 2.3-fold lower than the normal control (0.207 ± 0.013) (Figure 2B).

Thrombin clotting times for purified fibrinogen were 14.4 ± 0.2 sec for Sumida and 14.8 ± 0.7 sec for Sumida's mother, and were not significantly different from that of the normal control (14.2 ± 0.1 sec).

Scanning electron microscopy (SEM)

We observed fibrin clots under SEM in order to analyze differences in the ultrastructures of fibrin clots formed from fibrinogen of the normal control, Sumida, and Sumida's mother, (Figure 3). The density of clots formed from Sumida or Sumida's mother fibrinogen was higher than that of the normal control; however, the meshwork was curly, and fiber diameter was thinner than the normal control: 44.4 ± 8.1 nm for Sumida (n = 30, $p<0.01$), 39.0 ± 5.1 nm for Sumida's mother (n = 30, $p<0.01$), and 82.2 ± 8.5 nm for the normal control (n = 30).

Clot lysis assay

Many previous studies have shown that albumin binding variant fibrinogens lead to thrombosis; therefore, we performed the clot lysis assay to clarify whether clots were resistant to plasmin degradation *in vitro*. Lysis curves for the 2 sets of experimental conditions using the proposita's plasma fibrinogen are shown in Figure 4. The lag time, maximum slope (Lmax), and 50 % lysis time were measured on the clot lysis curve as described in the Materials and Methods. In the clot lysis of Sumida's fibrinogen initiated with the addition of plasmin, turbidity decreased after 10.0 ± 0.0 min (normal: 15.0 ± 0.0), and the 50 % lysis time was 55.0 ± 1.5 min (normal: 50.0 ± 2.8). These values were not significantly different from those of the normal control. In the clot lysis of Sumida's fibrinogen initiated with the t-PA/plasminogen mixture, the lag time and 50 % lysis time (45.0 ± 4.2 min and 81.0 ± 6.4 min, respectively) were also not significantly different from those of the normal control (42.0 ± 2.8 min and 77.5 ± 6.4 min, respectively).

Synthesis, secretion, and characterization of recombinant fibrinogens

Since the proposita's plasma fibrinogen antigenic concentration was 1.33 g/L, which

was less than the normal range, we analyzed the fibrinogen synthesis and secretion of AαC472S from CHO cell lines that expressed Aα472S- and Aα442S-fibrinogen as described in the Materials and Methods. AαC472 connects with AαC442 via a disulfide bond; therefore, we compared the synthesis and secretion of fibrinogen by Aα472S-CHO cells and Aα442S-CHO cells. In the normal fibrinogen-synthesizing cell line (AαN-CHO cells), the fibrinogen concentration was 1.80 ± 0.25 μg/mL (mean ± SD, n=10) for the culture medium and 2.23 ± 0.60 μg/mL for cell lysate, resulting in a culture medium to cell lysate ratio of 0.81 ± 0.16 (Figure 5). The fibrinogen concentration in the culture medium of each of the variant fibrinogen-expressing cell lines was 1.73 ± 0.23 μg/mL for Aα472S-CHO cells (n=10) and 2.88 ± 0.70 μg/mL for Aα442S-CHO cells (n=10, higher than AαN-CHO cells) (Figure 5A). The concentrations in the cell lysates were 1.85 ± 0.13 μg/mL for Aα472S-CHO cells and 2.75 ± 0.47 μg/mL for Aα442S-CHO cells (Figure 5B; significantly different $p<0.01$). The culture medium to cell lysate ratios were 0.94 ± 0.12 for Aα472S-CHO cells and 1.05 ± 0.24 for Aα442S-CHO cells, which was higher than that of AαN-CHO cells (no significance) (Figure 5C).

To confirm the purity of Aα472S- and Aα442S-fibrinogen purified from serum-free culture media, SDS-PAGE and Coomassie staining were performed under non-reducing

or reducing conditions (Figure 6). Under non-reducing conditions, intact fibrinogen, high molecular weight (HMW)- and low molecular weight (LMW)- fibrinogen [13], were observed in AαN-fibrinogen. Regarding Aα472S- and Aα442S-fibrinogen, the proportion of HMW-fibrinogen was lower than that of AαN-fibrinogen, whereas the proportion of LMW-fibrinogen was increased while that of low molecular weight prime (LMW')-fibrinogen [13] was significantly elevated (Figure 6A). These results were confirmed under reducing conditions. The proportion of the Aα-chains of Aα472S- and Aα442S-fibrinogen was lower than that of AαN-fibrinogen, namely the degradation of the Aα chains derived from variant fibrinogens was facilitated (Figure 6B). The degradation of the Aα chain derived from Aα442S-fibrinogen was faster than that from Aα472S-fibrinogen.

Discussion

We herein described a novel case of dysfibrinogenemia with a heterozygous missense mutation in *FGA* at exon 5, which resulted in an AαC472S substitution in the αC-domain and was designated as Sumida. Aα472C is an important residue for the formation of a disulfide bond between Aα442C. Sumida's purified plasma fibrinogen showed the presence of some albumin-binding variant fibrinogens and a dimer of a

variant fibrinogen. Although Sumida's fibrinogen concentration ratio of the thrombin time method to the antigenic method was 0.857 and notably higher than those observed for other γ-chain aberrant dysfibrinogenemias (0.05 – 0.50) reported previously [8, 14], thrombin-catalyzed fibrin polymerization using Sumida's purified plasma fibrinogen was markedly impaired with a less steep maximum slope and smaller final OD than those of the normal control.

We suspected that this patient may have hypodysfibrinogenemia because fibrinogen concentrations determined by both the functional and antigenic methods were lower than the lower level of reference intervals, whereas the fibrinogen concentration ratio was higher, as described above. To clarify the synthesis and secretion of fibrinogen by the proposita, we produced CHO cell lines that generated the recombinant variant fibrinogen, Aα472S or Aα442S, both of which are counterparts of the intra-chain disulfide bond in the Aα-chain. The culture medium to cell lysate ratios derived from both variant cell lines were unaffected while those of the Aα442S cell lines were higher than the normal control. These results were consistent with previous findings in COS-1 cells [15]. Namely, the destruction of the Aα442C-472C disulfide bond did not prevent the synthesis and secretion of fibrinogen. However, SDS-PAGE and the western blot analysis demonstrated that the degradation of the purified recombinant variant

fibrinogen from the culture media, Aα472S or Aα442S fibrinogen, was faster than that of the normal control; degradation of the Aα-chain was particularly enhanced. However, the same phenomenon was not observed for purified plasma fibrinogen from the proposita (Fig 1B). Therefore, we speculated that the variant Aα chain in Sumida's plasma fibrinogen was degraded faster than that of the normal control, and the Bβ and γ chains of the Aα chain-degraded variant fibrinogen also facilitated degradation by some plasma proteases, leading to a less constitutional proportion of variant to normal fibrinogen, which ultimately resulted in a higher fibrinogen concentration ratio of the thrombin time method to the antigenic method. Some of the cases of αC-domain-affected dysfibrinogenemias [16-32], including Lincoln (variant fibrinogen; 23%) [29], Caracas I (10 - 20%) [27], and Perth (12%) [31], were reported similarly to Sumida (Table 1).

The fibrin polymerization curve for Sumida's purified plasma fibrinogen showed markedly impaired lateral aggregation of protofibrils. The αC-domain has been shown to prompt the lateral aggregation of protofibrils during fibrin polymerization [6]. Therefore, the substitution of C to S at the Aα472 residue leads to the destruction of the disulfide bond, resulting in marked conformational changes in the αC-domain. Furthermore, our results demonstrated that disulfide bond-free Aα442C bound to the

high molecular weight proteins, mercapto-albumin or another variant fibrinogen (Aα442C). The αC-domain of the variant fibrinogen of Sumida exhibited weaker lateral aggregation due to structural failure and/or steric hindrance.

Excluding hypofibrinogenemias and amyloidgenic dysfibrinogenemias, 14 species of dysfibrinogenemias have been reported to date in the region of the αC-domain of the fibrinogen Aα chain [5]. Twelve out of 14 species were present as albumin-binding variant fibrinogens (10 species, 13 families) or the possibility of the presence of albumin-binding variant fibrinogens (2 species, 2 families) (Table 1) [16-32]. Six out of 13 families with the albumin-binding variant fibrinogen, affected the lateral aggregation of protofibrils, and thin fibrin fiber diameters manifested thrombotic complications (Table 1), whereas 4 out of 13 families manifested bleeding complications (Table 1). Sumida's fibrinogen also affected the lateral aggregation of protofibrils and caused a thin fibrin fiber diameter. Therefore, we performed a clot lysis assay by the procedure involving plasmin or a mixture of t-PA and plasminogen being overlaid onto the clot. Sumida's clot lysis was similar to that of the normal control under both conditions; however, it is difficult to evaluate whether Sumida's clot structure was resistant to plasmin digestion and Sumida's fibrin activated plasminogen mediated by t-PA. The activation of plasminogen mediated by t-PA is effectively enhanced during fibrin

formation [33]; therefore, plasminogen is not activated in clots formed in advance. On the other hand, impaired plasminogen activation has already been reported in several aberrant fibrinogens, namely Caracas V [17], Dusart [18,19], Chapel Hill [20], Marburg[25], and Guarenas [26] (Table 1). If impaired clot lysis (fibrinolysis) proves to be a significant aspect of thrombosis in these variants, it may be related to the loss or steric hindrances of high-affinity plasminogen and t-PA binding sites on the variant Aα chain of fibrin [34].

Sumida's fibrinogen is a novel heterozygous dysfunctional fibrinogen characterized by the substitution of Ser for Aα472Cys, the existence of additional disulfide-bonded molecules, impairment of lateral aggregation of protofibrils, and formation of thin fiber fibrin clots. Although the proposita and her affected mother had no history of bleeding and a thrombotic tendency, both must be cautious not only of bleeding, but also thrombotic complications because some similar variants located in the αC-domain exhibited impairments in t-PA-mediated plasminogen activation.

Authorship

M Ikeda performed the research, analyzed the data, and wrote the manuscript. S Arai, S Mukai, Y Takezawa, F Terasawa , and N Okumura designed the research and discussed

the data, and N Okumura reviewed the manuscript.

Conflicts of Interest Statement

The authors state that they have no conflicts of interest.

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References

[1] Doolittle RF, Bouma IH, Cottrell BA, Strong D, Watt KWK. The covalent structure of human fibrinogen. In: Bing DH, editor. The chemistry and physiology of the human plasma proteins, New York: Pergamon Press; 1979, p.77-95.

[2] Doolittle RF. Fibrinogen and fibrin. Ann Rev Biochem 1984;53:195-229.

[3] Weisel JW. Fibrinogen and fibrin. Adv Protein Chem 2005;70:247-99.

[4] Weisel JW, Veklich Y, Gorkun OV. The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. J Mol Biol 1993;232:285-97.

[5] Groupe d‟Etude sur l‟Hémostase et la Thrombose. “ Base de données des variants du Fibrinogène”. GEHT Web site, <http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Basede-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html>, (updated on 26/01/2012)

[6] Gorkun OV, Veklich YI, Medved LV, Henschen AH, Weisel JW. Role of the αC domains of fibrin in clot formation. Biochemistry 1994;33:6986-97.

[7] Collet JP, Moen JL, Veklich YI, Gorkun OV, Lord ST, Montalescot G, Weisel JW. The αC domains of fibrinogen affect the structure of the fibrin clot, its physical

properties, and its susceptibility of fibrinolysis. Blood 2005;106:3824-30.

[8] Okumura N, Furihata N, Terasawa F, Nakagoshi R, Ueno I, Katsuyama T. Fibrinogen Matsumoto I: A γ364Asp → His (GAT→CAT) substitution associated with defective fibrin polymerization. Thromb Haemost 1996;75:887-91.

[9] Ikeda M, Kobayashi T, Arai S, Mukai S, Takezawa Y, Terasawa F, et al. Recombinant γT305A fibrinogen indicates severely impaired fibrin polymerization due to the aberrant function of hole 'a' and calcium binding sites. Thromb Res 2014;134:518-25.

[10] Kamijyo Y, Hirota-Kawadobora M, Yamauchi K, Terasawa F, Honda T, Ikeya M, et al. Analysis of plasmin generation and clot lysis of plasma fibrinogen purified from heterozygous dysfibrinogenemia, BβGly15Cys (Hamamatsu II). Blood Coagul Fibrinolysis 2009;20:726-32.

[11] Kobayashi T, Arai S, Ogiwara N, Takezawa Y, Nanya M, Terasawa F, et al. γ375Wfibrinogen-synthesizing CHO cells indicate the accumulation of variant fibrinogen within endoplasmic reticulum. Thromb Res 2014;133:101-7.

[12] Okumura N, Gorkun OV, Lord ST. Severely impaired polymerization of recombinant fibrinogen γ-364 Asp→His, the substitution discovered in a heterozygous individual. J Bio Chem 1997;272:29596-601.

[13] Holm B, Nilsen DWT, Kierulf P, Godal HC. Purification and characterization of 3 fibrinogens with different molecular weights obtained from normal human plasma. Thromb Res 1985;37:165-76.

[14] Ikeda M, Arai S, Mukai S, Inaba T, Okumura N. Comparison of fibrin polymerization between heterozygous plasma fibrinogen, γF322S, and recombinant γ322S fibrinogen. J Jap Soc Lab Hematol (Kensa Ketsueki) 2014;15:163-70. (in Japanese)

[15] Zhang JZ, Redman C. Fibrinogen assembly and secretion. Role of intrachain disulfide loops. J Biol Chem 1996;271:30083-8.

[16] Hanss M, Vergnes C, Rugeri L, French P, DE Mazancourt P. A new electrophoretic variant of fibrinogen associated with venous thromboembolism, fibrinogen Bordeaux Aα Arg439→Cys. J Thromb Haemost 2008;6:1422-4.

[17] Marchi R, Mirshahi SS, Soria C, Mirshahi M, Zohar M, Collet JP, et al. Thrombotic dysfibrinogenemia. Fibrinogen “Caracas V” relation between very tight fibrin network and defective clot degrability. Thromb Res 2000;99:187-93.

[18] Collet JP, Soria J, Mirshahi M, Hirsch M, Dagonnet FB, Caen J, et al. Dusart syndrome: a new concept of the relationship between fibrin clot architecture and fibrin clot degradability: hypofibrinolysis related to an abnormal clot structure. Blood

1993;82:2462-9.

[19] Lijnen HR, Soria J, Soria C, Collen D, Caen JP. Dysfibrinogenemia (fibrinogen Dusart) associated with impaired fibrin enhanced plasminogen activation. Thromb Haemost 1984;51:108-9.

[20] Wada Y, Lord ST. A correlation between thrombotic disease and a specific fibrinogen abnormality (A alpha 554 Arg → Cys) in two unrelated kindred, Dursat and Chapel Hill III. Blood 1994;84:3709-14.

[21] Tarumi T, Martincic D, Thomas A, Janco R, Hudson M, Baxter P, et al. Familial thrombophilia associated with fibrinogen Paris V: Dusart syndrome. Blood 2000;96:1191-3.

[22] Furlan M, Steinmann C, Jungo M, Bögli C, Baudo F, Redaelli R, et al. A frameshift mutation in exon V of the Aα-chain gene leading to truncated Aα -chains in the homozygous dysfibrinogen Milano III. J Biol Chem 1994;269:33129-34.

[23] Sugo T, Nakamikawa C, Takebe S, Kohno I, Egbring R, Matsuda M. Factor XIIIa cross-linking of the Marburg fibrin: formation of αm•γn-heteromultimers and the α-chain-linked albumin•γ complex, and disturbed protofibril assembly resulting in acquisition of plasmin resistance relevant to thrombophilia. Blood 1998;91:3282-8.

[24] Collen A, Maas A, Kooistra T, Lupu F, Grimbergen J, Haas FJ, et al. Aberrant

fibrin formation and cross-linking of fibrinogen Nieuwegein, a variant with a shortened Aα-chain, alters endothelial capillary tube formation. Blood 2001;97:973-80.

[25] Koopman J, Haverkate F, Grimbergen J, Egbring R, Lord ST. Fibrinogen Marburg: a homozygous case of dysfibrinogenemia, lacking amino acid Aα 461-610 (Lys 461 AAA→stop TAA). Blood 1992;80:1972-9.

[26] Brennan SO, Mosesson MW, Lowen R, Frantz C. Dysfibrinogenemia (fibrinogen Wilmington) due to a novel Aα chain truncation causing decreased plasma expression and impaired fibrin polymerization. Thromb Haemost 2006;96:88-9.

[27] Marchi R, Meyer M, de Bosch N, Soria J, Arocha-Piñango CL, Weisel JW. Biophysical characterization of fibrinogen Caracas I with an Aα-chain truncation at Aα -466 Ser: identification of the mutation and biophysical characterization of properties of clots from plasma and purified fibrinogen. Blood Coagul Fibrinolysis 2004;15:285-93.

[28] Marchi R, Carvajal Z, Meyer M, Soria J, Ruiz-Saez A, Arocha-Piñango CL, et al. Fibrinogen Guarenas, an abnormal fibrinogen with an Aα -chain truncation due to a nonsense mutation at Aα 467 Glu (GAA) →stop (TAA). Thromb Res 2006;118:637-50.

[29] Ridgway HJ, Brennan SO, Gibbons S, George PM. Fibrinogen Lincoln: a new truncated α chain variant with delayed clotting. Br J Haematol 1996;93:177-84.

[30] Dempfle CE, George PM, Borggrefe M, Neumaier M, Brennan SO. Demonstration of heterodimeric fibrinogen molecules partially conjugated with albumin in a novel dysfibrinogen: fibrinogen Mannheim V. Thromb Haemost 2009;102:29-34.

[31] Homer VM. Mullin JL, Brennan SO, BarrA, George PM. Novel Aα chain truncation (fibrinogen Perth) resulting in low expression and impaired fibrinogen polymerization. J Thromb Haemost 2003;1:1245-50.

[32] Margaglione M, Vecchione G, Santacroce R, D'Angelo F, Casetta B, Papa ML, et al. A frameshift mutation in the human fibrinogen Aα-chain gene (Aα [499] Ala frameshift stop) leading to dysfibrinogen San Giovanni Rotondo. Thromb Haemost 2001;86:1483-8.

[33] Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem 1982;257:2912-9.

[34] Tsurupa G, Medved L. Identification and characterization of novel tPA- and plasminogen-binding sites within fibrin(ogen) αC-domains. Biochemistry 2001;40:801-8.

Table legend

Table 1. List of variant fibrinogens with mutations located in the αC-domain and presented as albumin-binding forms.

Abbreviations; T: thrombosis, B: bleeding, A: asymptomatic, WB: western blot analysis, HPLC: high performance liquid chromatography, ND: not determined, NA: not applicable.

Numbers in parentheses are functional/protein fibrinogen concentration ratios derived from homozygous patients.

Figure legends

Figure 1. SDS-PAGE and Western blot analysis of Sumida's plasma fibrinogen.

Coomassie brilliant blue-stained 10% SDS-PAGE run under reducing conditions (A) and 5% SDS-PAGE run under non-reducing conditions (B). Approximately 5 μg fibrinogen was applied to each lane for panel A and 7 μg for panel B. The proteins that were transferred to a nitrocellulose sheet from 5% SDS-PAGE run under non-reducing conditions were visualized with a rabbit antihuman fibrinogen antibody (C) or a rabbit antihuman albumin antibody (D). Approximately 200 ng fibrinogen was applied to each lane for panel C and 400 ng for panel D. Lane 1: normal control, lane 2: Sumida, lane 3: Sumida's mother, lane M: molecular marker.

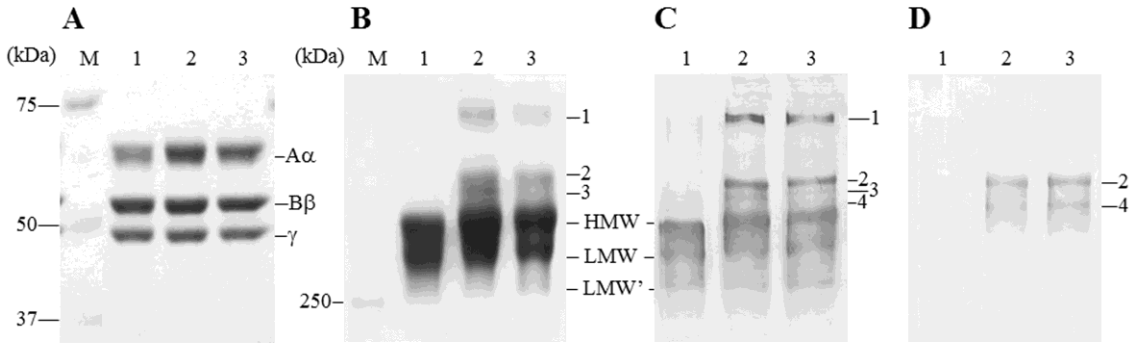

A
(kDa)
M
1
2
3
75—
50—
37—
–Aα
–Bβ
–γ
B
(kDa)
M
1
2
3
250–
–1
–2
–3
– HMW –
– LMW –
– LMW' –
C
1
2
3
—1
—2
—3
—4
D
1
2
3
—2
—4

Figure 2. Thrombin catalyzed fibrin polymerization.

Polymerization of fibrinogen (0.2 mg/mL) was initiated with thrombin (0.05 U/mL; A, 0.5 U/mL; B), and the change in turbidity with time was followed at 350 nm for normal (●), Sumida (◆), Sumida's mother (◇) in HBS buffer. Polymerization was performed in triplicate and each representative curve is depicted.

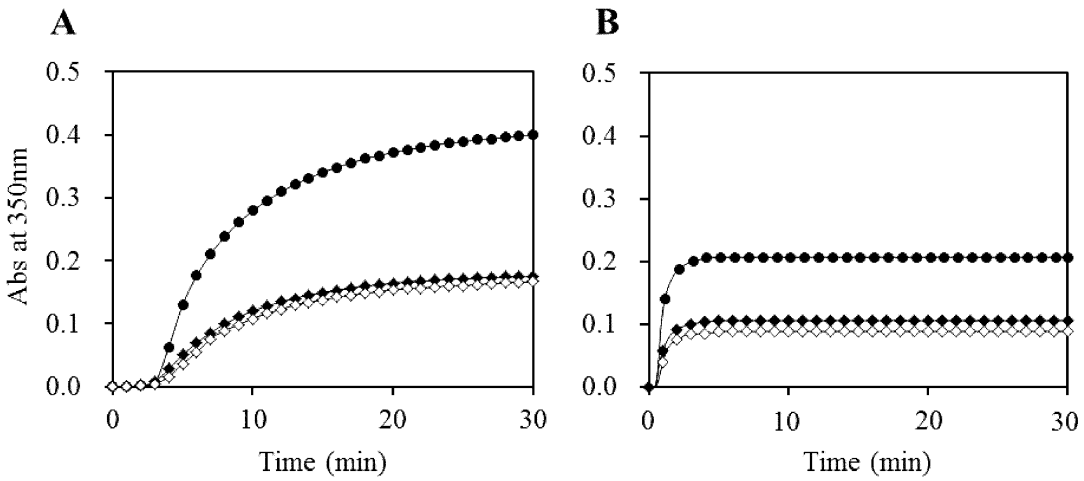


Figure 3. Scanning electron microscopy (SEM) of fibrin clots.

All samples were prepared as described in Materials and methods. Micrographs were taken at 3,000x (normal control; A, Sumida; B, Sumida's mother; C) and 20,000x (normal control; D, Sumida; E, Sumida's mother; F). White bars represent 10μm in A-C and 1.0μm in D-F.

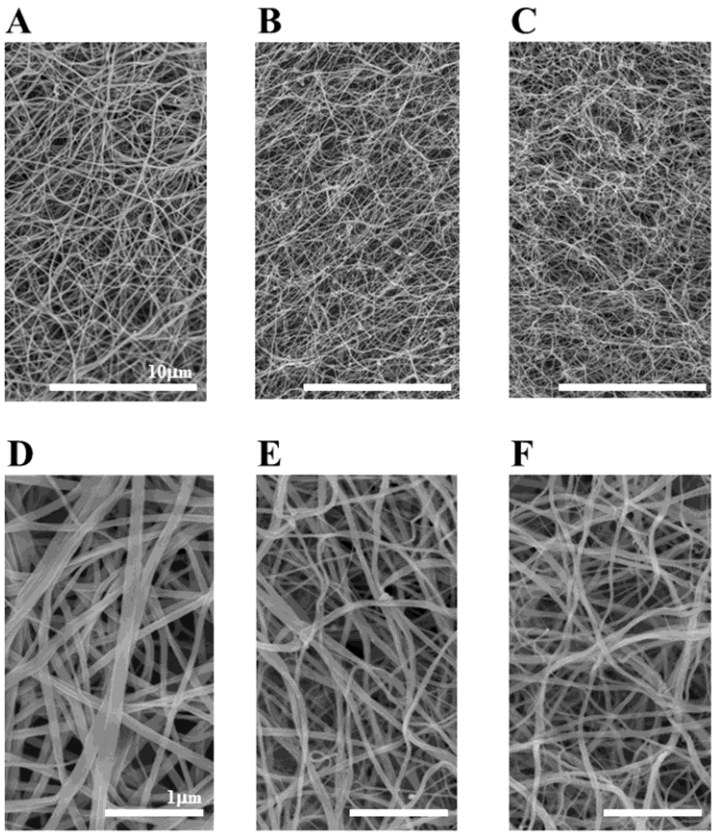


Figure 4. Lysis of fibrin clots made from plasma fibrinogens.

After the completion of fibrin polymerization, either plasmin (A) or a mixture of t-PA and plasminogen (B) was overlaid onto the clots as described in Materials and methods. Clot lysis was performed in triplicate and each representative curve is shown as the normal (●) and Sumida (○).

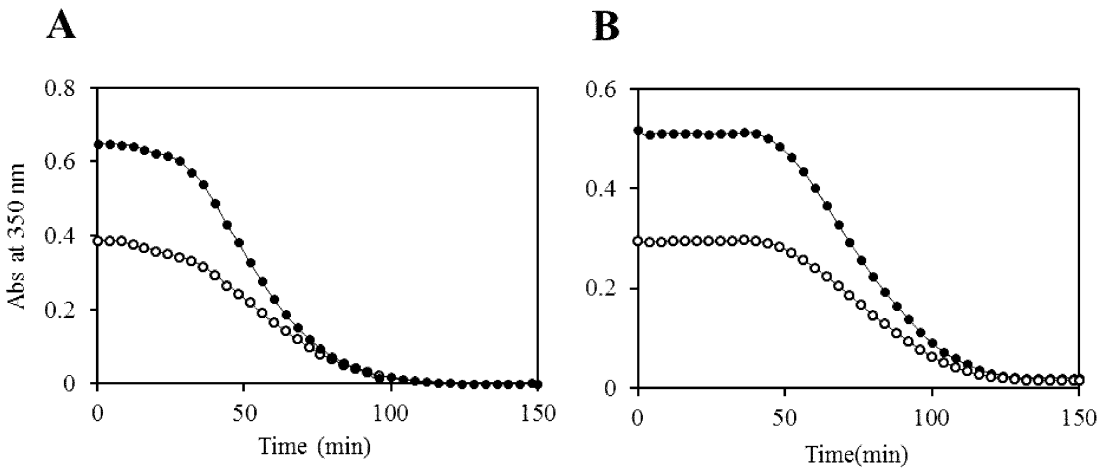


Figure 5. Synthesis and secretion of variant fibrinogens in transfected CHO cells.

The concentration of fibrinogen in the culture media (A) and cell lysates (B) were measure by ELISA as described in Materials and Methods. The ratio of fibrinogen concentration in the culture media to that of cell lysates is shown in panel C. All cell lines are used 10 clones and the mean value was presented with standard deviations indicated by error bars. AαN: normal control (wild type) cells, Aα472S: Aα472S cells, and Aα442S: Aα442S cells. *Significantly different between Aα472S and Aα442S cell lines ($p<0.01$).

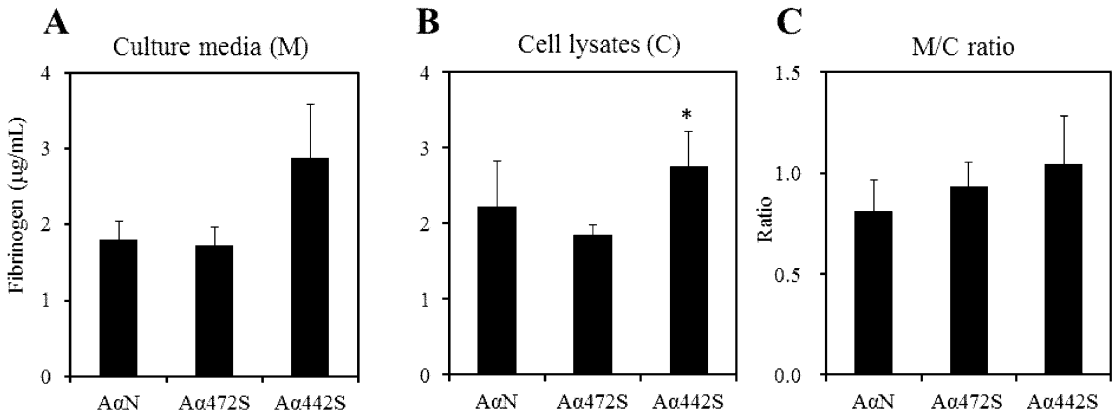
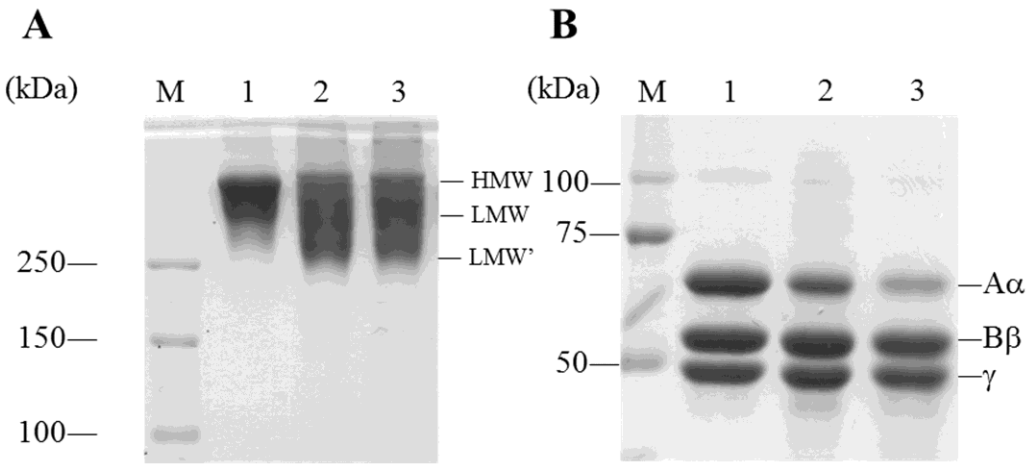


Figure 6. Characterization of recombinant fibrinogens.

Coomassie brilliant blue-stained 10% SDS-PAGE run under non-reducing conditions (A) and 5% SDS-PAGE under reducing conditions (B). Each lane was applied approximately 7 μg fibrinogen for panel A and 9 μg for panel B. Lane 1: recombinant AαN, lane 2: Aα472S, lane 3: Aα442S, lane M: molecular marker.



case	Variant (amino acid)	genotype	symptom	functional fibrinogen (mg/dL)	fibrinogen protein (mg/dL)	functional/ protein ratio	Albmin binding	percentage of variant Aα chain	polymeri-zation	fiber diameter	tPA-plasminogen clot lysis	tPA-mediated plasmin activation	references
Bordeaux	Aα439R>C	hetero	T	210	195	1.08	(+) 439C-Alb	ND	low	ND	normal	ND	16
Caracas V	Aα532S>C	hetero	T	530	ND	NA	ND	ND	low	thin	delay	delay	17
Dusart	Aα554R>C	hetero	T	165	190	0.87	(+) 554C-Alb	ND	low	thin	delay	delay	18, 19
Chapel Hill	Aα554R>C	hetero	T	258	332	0.78	(+) 554C-Alb	ND	low	thin	ND	delay	20
Nashville	Aα554R>C	hetero	T	258	ND	NA	(+) 554C-Alb	ND	low	ND	ND	ND	21
		hetero	T	306	406	0.75	(+) 554C-Alb	ND	low	ND	ND	ND	
Milano III	Aα452flameshift, 454Stop	homo	T	20	260	(0.08)*	(+) 442C-Alb	NA	low	ND	ND	ND	22,23
		hetero	A	210	310	0.68	(+) 442C-Alb	lower (WB)	low	ND	ND	ND	
Nieuwegein	Aα453flameshift, 454Stop	homo	A	ND	170	NA	(+) 442C-Alb	NA	low	thin	ND	ND	24
Marburg	Aα460K>Stop	homo	B+T	<25	60	(<0.42)*	(+) 442C-Alb	NA	low	ND	delay	delay	25
		hetero	A	ND	170	NA	ND	lower (WB)	ND	ND	ND	ND	

Table1

Wilmington	Aα465flameshift, 478Stop	hetero	B	140	230	0.61	(+) 442C-Alb	lower (HPLC)	low	ND	ND	ND	26
Caracas I	Aα467E>Stop	hetero	B	320	286	1.12	(+) 442C-Alb	10-20%	low	thin	ND	ND	27
Guarenas	Aα467E>Stop	hetero	B	ND	250	NA	(+) 442C-Alb	ND	low	thin	ND	delay	28
Lincoln	Aα475flameshift (476C), 479Stop	hetero	B	190	ND	NA	(-)	23%	low	ND	ND	ND	29
Mannheim V	Aα494flameshift (517C), 518Stop	hetero	A miscarriages	1.78	300	0.59	(+) 517C-Alb	ND	low	ND	ND	ND	30
Perth	Aα495flameshift (517C), 518Stop	hetero	B	180	330	0.55	(+) 517C-Alb	12%	low	ND	normal	ND	31
San Giovanni Rotondo	Aα499flameshift (517C), 518Stop	hetero	A	73	226	0.32	(+) 517C-Alb	lower (HPLC)	low	ND	ND	ND	32

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