

Mutation Report

**Heterozygous B β C-terminal 12 amino acid-elongation variant, B β X462W (Kyoto VI),
showed dysfibrinogenemia**

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Abstract

Objective A heterozygous patient with dysfibrinogenemia with slight bleeding and no thrombotic complications was diagnosed with fibrinogen Kyoto VI (K-VI). To elucidate the genetic mutation(s) and characterize the variant protein, we performed the following experiments and compared with identical and similar variants that have already been reported.

Methods The proposita's PCR-amplified DNA was analyzed by sequencing and her purified plasma fibrinogen underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting, fibrin polymerization, and scanning electron microscopic observation of fibrin clot and fibers.

Results Sequence analyses showed that K-VI fibrinogen substituted W (TGG) for terminal codon (TAG), resulting in 12 amino acid elongation 462-473 (WSPIRRFLFCM) in the B β -chain. Protein analyses indicated that the presence of some albumin-binding variant fibrinogens and a dimeric molecule of variant fibrinogens reduced fibrin polymerization, with a thinner fiber and aberrant fibrin network. These results are almost the same as for the identical variant of Magdeburg; however, different from the similar variant of Osaka VI [12 amino acid elongation 462-473 (KSPIRRFLFCM) in the B β -chain] in the presence of variant forms and clot structure.

Conclusion We speculate the side-chain difference at 462 residues, W in K-VI and K in Osaka VI, and/or the difference in the presence of disulfide bridged forms of variant fibrinogens, leading to the notable difference in the fibrin bundle network. Although a strong evolutionary and structural association between B β - and γ -chain molecules is established, the corresponding recombinant 15-residue elongation variants of the fibrinogen γ -chain showed reduced assembly and secretion.

Introduction

Fibrinogen is a 340 kDa plasma hexameric glycoprotein composed of two sets of three different polypeptide chains ($A\alpha$: 610, $B\beta$: 461, and γ : 411 residues) [1, 2]. Conversion of fibrinogen to insoluble fibrin plays a pivotal role in blood coagulation and thrombosis. During blood coagulation, thrombin cleaves fibrinopeptide A and B from fibrinogen to form fibrin monomers and these spontaneously polymerize, and finally form an insoluble fibrin clot consisting of a multi-stranded and branched fiber network [3].

Inherited fibrinogen disorders causing quantitative and/or qualitative alterations of this molecule may result in bleeding, thrombotic, or asymptomatic phenotypes. As many as 300 families with dysfunctional (qualitative) fibrinogens have been analyzed genetically and/or structurally. These are listed on the GEHT homepage [4] (updated on 01/02/2011, http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html). Most of these variants are present in either the $A\alpha$ -chain or the γ -chain, while variants of the $B\beta$ -chain have been found in 34 families throughout the world.

We found a female patient with dysfibrinogenemia with slight bleeding and no thrombotic complications who was diagnosed with fibrinogen Kyoto VI (K-VI), named in accordance with her place of residence. An identical variant has already been reported as fibrinogen Magdeburg [abstract #T22-48, XVIIth International Fibrinogen Work Shop, 2002] and a similar variant was also reported as fibrinogen Osaka VI [5]. It is interesting that the difference between K-VI and Osaka VI fibrinogen was only the residue at 462 residues, W and K, respectively. In this report, we analyzed the protein characterization and function of K-VI fibrinogen and fibrin clot structure derived from K-VI fibrinogen, and compared these with reports of fibrinogen Magdeburg and Osaka VI.

Patient and experimental procedures

The proposita of K-VI was a 62-year-old female who showed a low level of plasma fibrinogen at coagulation screening tests for hip replacement surgery. Although she had vaginally delivered two children and had suffered postpartum bleeding requiring blood transfusion at the first delivery, both children showed a normal level of plasma fibrinogen, and the two children and other family members had no bleeding tendencies or thrombotic complications. With informed consent, blood collection and separation of plasma were performed as previously described [6]. Separated plasma was used for coagulation tests and purification of fibrinogen, and buffy coat cells were extracted to prepare genomic DNA. This analysis was approved by the Ethics Committee for Genetic Analysis of Shinshu University School of Medicine. Unfortunately, we did not analyze the presence of dysfibrinogenemia in her family.

Polymerase chain reaction (PCR) amplification and DNA sequencing of all exons and exon-intron boundaries in the fibrinogen A α -chain, B β -chain, and γ -chain genes were performed as described elsewhere [7].

The blood coagulation screening tests and the immunologically determined fibrinogen concentration were performed as described elsewhere [8]. After informed consent, plasma fibrinogen was purified from the proposita and a normal control subject (NC) using immunoaffinity chromatography [9].

Purified fibrinogen was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing (5% polyacrylamide gel) or reducing conditions (10% polyacrylamide gel) and stained with Coomassie brilliant blue. Another non-reducing (5% polyacrylamide gel) was followed by immunoblot analysis and used with a

rabbit antihuman fibrinogen antibody (Dako, Carpinteria, CA, USA) or a rabbit antihuman albumin antibody (Dako) with the reacting species being visualized with the aid of alkaline phosphatase-conjugated goat antirabbit IgG antibody (EY Laboratories Inc., San Mateo, CA USA) [6].

Thrombin-catalyzed fibrin polymerization was performed as described elsewhere [6] with minor modification. Clottability was performed and calculated as described [10].

Samples for scanning electron microscopy were prepared as described previously [6] with minor modification. Briefly, the clots were fixed in 2.5 % glutaraldehyde overnight, stained with 1.0 % osmium tetroxide, freeze-dried (JFD-310 freeze drying device; Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan), mounted, osmium plasma-coated at 5 nm thickness in Neo-AN (Meiwafosis Co. Ltd., Tokyo, Japan), and finally viewed on a JSM-6510LV (Japan Electron Optics Laboratory Co. Ltd).

Results

Coagulation tests

The proposita's plasma fibrinogen concentration was much lower when determined by the thrombin-time method (0.75 g/l) than when determined by the immunological method (2.35 g/l). The proposita's prothrombin time (12.5 sec) and activated partial thromboplastin time (40.9 sec) were each slightly longer than their reference range (10.0–12.0 sec and 23.0–38.0 sec, respectively).

DNA sequence analysis of the fibrinogen gene of the propositus

Sequence analysis for PCR amplified fragment of B β -chain exon 8 indicated the missense mutation g.7670A >G (nucleotide positions were numbered taking the starting point of the

transcription of the B β -chain gene as 1) (NCBI accession number NG 008833) in a heterozygous state, causing substitution of W (TGG) for terminal codon (TAG) and resulting in 12 amino acid elongation 462-473 (WSPIRRFLFCM) in the B β -chain. Except known polymorphic positions, no mutations leading dysfibrinogenemia were found in other regions of fibrinogen B β -chain and A α - and γ -chain (data not shown).

Characterization of Kyoto-VI fibrinogen

The gel resolved under non-reducing conditions and stained by Coomassie revealed that the normal pattern shows three bands, corresponding to high molecular weight (340 kDa; HMW), low molecular weight (305 kDa; LMW), and low molecular weight prime (270 kDa; LMW') fibrinogen fractions [11], while the proposita's fibrinogen had three minor extra bands larger than the HWM fibrinogen fraction (Figure 1B).

To identify these bands, plasma was separated by SDS-PAGE run under non-reducing conditions followed by immunoblot analysis using a rabbit antihuman fibrinogen antibody (Figure 1C) or a rabbit antihuman albumin antibody (Figure 1D). Since there is no appropriate higher molecular weight marker other than 250 kDa, the precise relative molecular mass of band was not estimated. For visualizing with anti-fibrinogen antibody, the proposita's fibrinogen had four minor extra bands larger than the HWM-fibrinogen fraction (Figure 1C; numbered as Band-1–Band-4, respectively), whereas for visualizing with anti-albumin antibody, the proposita's fibrinogen had three extra bands (Figure 1D; numbered Band-3–Band-5, respectively). Band-1 and Band-2 only reacted with the rabbit antihuman fibrinogen antibody, and we speculate that these bands are a dimeric molecule of variant HMW fibrinogen and variant LMW fibrinogen, respectively (Figure 1C and 1D). On the other hand, we also speculate that Band-3 is a complex of variant HMW fibrinogen and two

albumin molecules, Band-4 is a complex of variant HMW fibrinogen and albumin and/or a complex of variant LMW fibrinogen and two albumin molecules, and finally Band-5 is a complex of variant LMW fibrinogen and albumin.

Thrombin-catalyzed fibrin polymerization and clottability

We found that polymerization of K-VI fibrinogen was impaired compared with that of NC (Figure 2) ; namely, it demonstrated a slightly but significantly longer lag period (177 ± 4 sec), a slower maximum slope (0.80 ± 0.01 Abs $\times 10^{-3}$ /sec), and smaller 30-min Δ absorbance (0.518 ± 0.013 Abs) than NC (126 ± 9 sec, 4.50 ± 0.18 Abs $\times 10^{-3}$ /sec, and 1.115 ± 0.075 Abs, respectively) (three parameters; Student's *t*-test, $p < 0.05$). In addition, the clottability of K-VI fibrinogen, i.e., the percentage of fibrinogen used for clot formation, was 96.3 ± 0.1 %, which was significantly different from that of the normal control (99.1 ± 0.6 %) ($n=3$, Student's *t*-test, $p < 0.05$), but was not markedly reduced.

Observation of fibrin clots and fibers by scanning electron microscopy

For the K-VI clot, the fibrin fiber network was irregular and the density of the bundles of fibrin fibers was lower (Figure 3 A and 3B, at a magnification of 3,000 \times), and the fiber diameters were significantly thinner in the K-VI clot (91.8 ± 28.6 nm) than in the NC clot (129.8 ± 34.7 nm) ($n=60$, Student's *t*-test, $p < 0.001$) (Figure 3C and 3D, at a magnification of 20,000 \times).

Discussion

In this report, we identified a heterozygous dysfibrinogenemia, Kyoto VI, 12 amino acid elongation 462-473 (WSPIRRFLFCM) variant in the B β -chain. The same heterozygous

variant was found in a female patient with slight bleeding and recurrent spontaneous abortions and reported as fibrinogen Magdeburg II in Germany [abstract #T22-48, XVIIth International Fibrinogen Work Shop, 2002]. Moreover, a similar variant was reported as fibrinogen Osaka VI, heterozygous 12 amino acid elongation 462-473 (KSPIRRFLLFCM) variant in the B β -chain [5]. Plasma fibrinogen from K-VI showed the presence of albumin-binding variant fibrinogens and the dimeric molecule of variant fibrinogens, these being similar to the observation of Magdeburg II plasma fibrinogen, whereas Osaka VI plasma fibrinogen showed only the presence of dimeric molecules of variant fibrinogens and the absence of albumin-binding variant fibrinogens. Although we cannot explain the difference, these disulfide bridged forms of three variant fibrinogens might be caused by the presence of 472Cys at the 11th residue of 12 elongation residues.

In addition, K-VI plasma fibrinogen showed impairment of lateral aggregation during thrombin-catalyzed fibrin polymerization and K-VI fibrin clots formed with thrombin, showing an irregular fibrin fiber network of fibrin, with a lower density of the bundles of fibrin fibers and a smaller fiber diameter in this case. In contrast, Osaka VI plasma fibrinogen showed markedly reduced lateral aggregation of fibrin monomer polymerization, and Osaka VI fibrin clots formed with thrombin, showing large pores bounded by smaller secondary, highly branched lacelike networks. We speculate that a side-chain difference at 462 residues, W in K-VI and K in Osaka VI, and/or the difference of the presence of disulfide bridged forms of variant fibrinogens, led to the notable difference in the fibrin bundle network [5].

In conclusion, the naturally occurring heterozygous Kyoto VI variant fibrinogen, 12 amino acid elongation 462-473 variant in the B β -chain, showed dysfibrinogenemia with the presence of disulfide bridged forms of variant fibrinogens, reduced fibrin polymerization, and a thinner fiber and aberrant fibrin network.

Disclosure of Conflicts of Interest

The authors state that they have no conflicts of interest.

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Figure legends

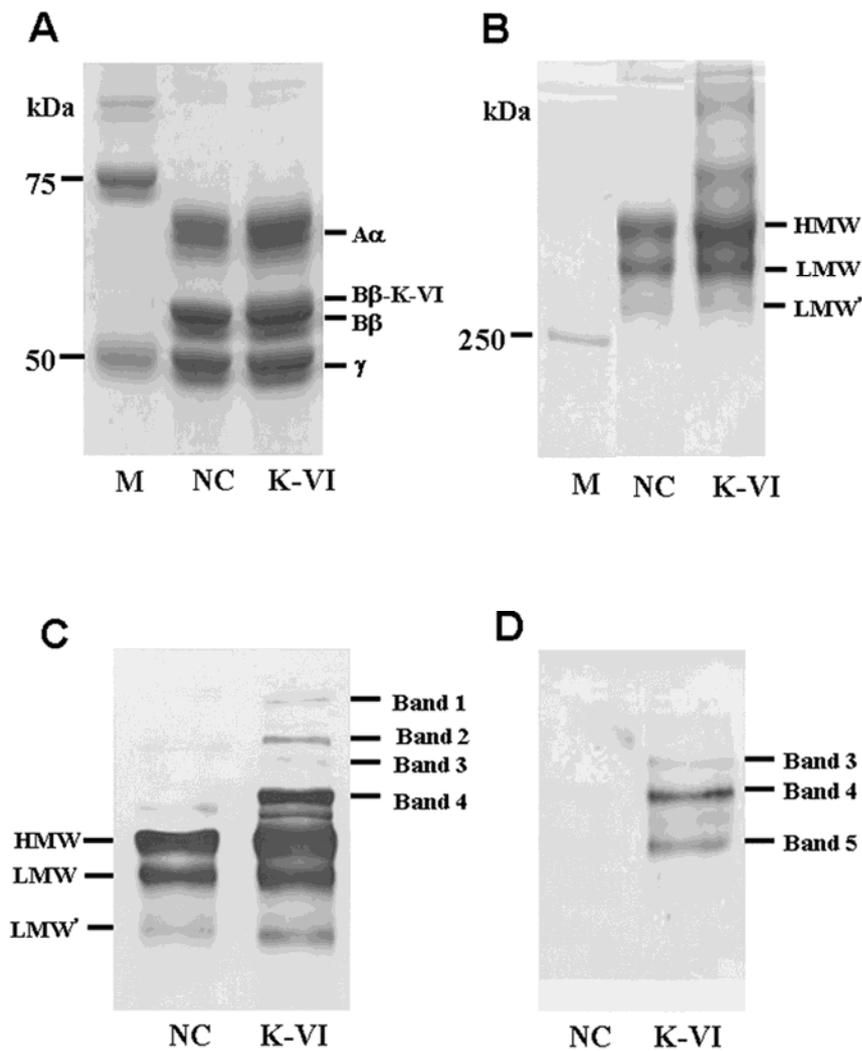


Figure 1. SDS-PAGE analysis and Western blot analysis of Kyoto VI fibrinogen. (A) Coomassie brilliant blue-stained 10% SDS-PAGE run under reducing conditions and **(B)** 5% SDS-PAGE run under non-reducing conditions. The proteins that were transferred to a

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). HMW, LMW, and LMW' are the high molecular weight, low molecular weight, and low molecular weight prime fibrinogen fractions, respectively. The extra bands observed for Kyoto VI are designated as Band-1 to Band-5. The lanes are for normal control fibrinogen (NC), Kyoto VI (K-VI), and the molecular markers (M).

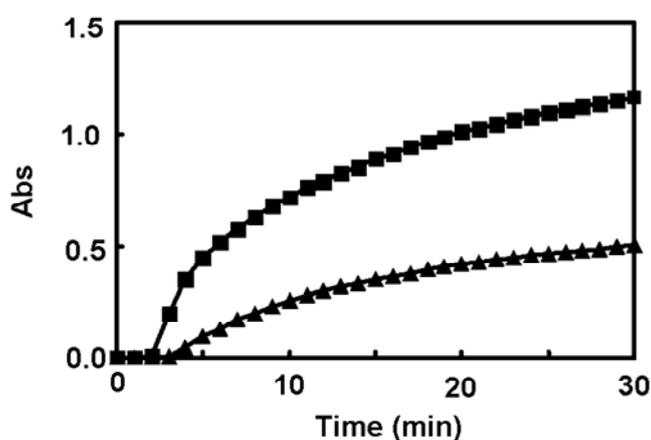


Figure 2. Thrombin-catalyzed fibrin polymerization. Thrombin-catalyzed fibrin polymerization was monitored at ambient temperature and 350 nm. Normal control (■) and Kyoto VI (▲). The final concentrations of fibrinogen and thrombin were 0.45 mg/ml and 0.05 U/ml, respectively, in *N*-[2-hydroxy-ethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) buffer at pH 7.4 and 0.12M NaCl containing 1.0 mM CaCl₂.

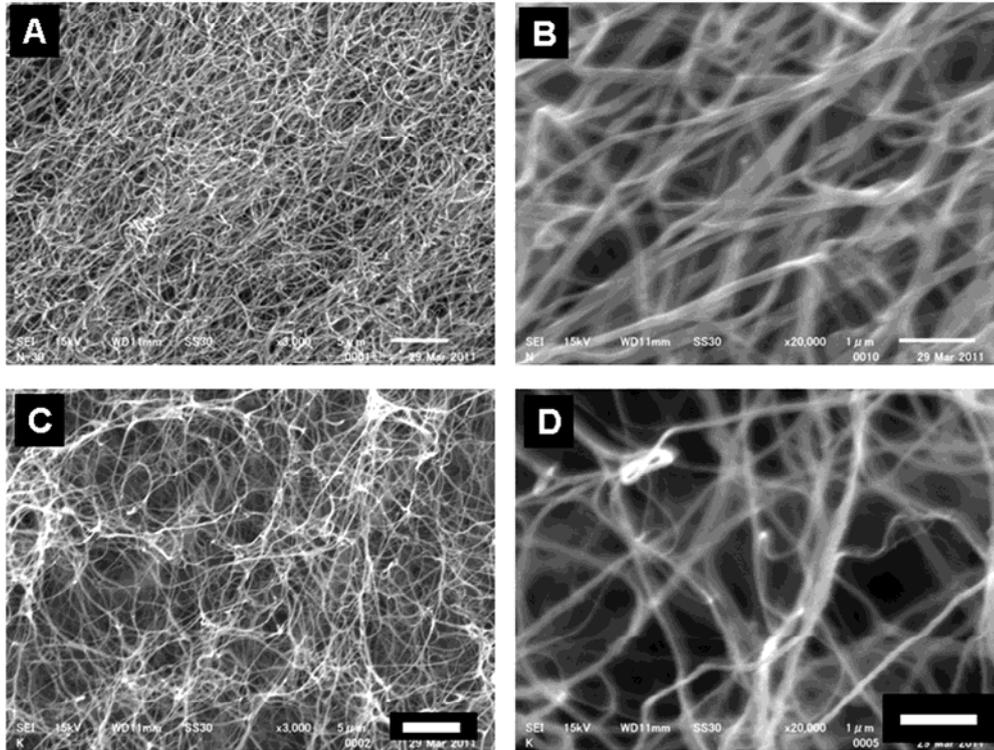


Figure 3. Scanning electron microscopic images of fibrin clots. The images were taken at 3,000 \times for (A) and (C), and 20,000 \times for (B) and (D) with a 15.0 kV accelerating voltage. A and B show normal control clots, and C and D show Kyoto VI clots. The white bar in black box represents 5 μm at 3,000 \times and 1 μm at 20,000 \times .