A novel frameshift mutation in the fibrinogen γC terminal region,

FGG c.1169_1170 del AT, leading to hypofibrinogenemia

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Introduction

Fibrinogen is a 340-kDa plasma glycoprotein that is a hexamer containing two copies of three different polypeptide chains: Aα, Bβ, and γ [1]. The γ-chain has two different forms: the predominant form (γA) contains 411 residues and the variant form (γ') consists of 427 residues [2]. Aα-, Bβ-, and γA-chains are coded by *FGA* (five exons), *FGB* (eight exons), and *FGG* (10 exons), respectively, while the γ'-chain is produced by alternative splicing, resulting in the translation of exons 1-9 of *FGG* [3]. The γA- and γ'-chains, hereafter referred to as γ- and γ'-chains, respectively, have the same residues from 1 to 407, while residues after 409 differ. More than 350 different mutations in *FGA*, *FGB*, and *FGG* have been reported and classified with four different manifestations: afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, and hypodysfibrinogenemia [4]. Hypofibrinogenemia has been defined as reduced levels of both functionally and immunologically determined fibrinogen in plasma.

We recently identified a novel heterozygous variant (designated as Ota I) with the deletion of AT (c.1169_1170) in *FGG* exon 9 in a hypofibrinogenemic patient. This nucleotide mutation leads to a frameshift (FS) mutation starting at codon 364 and results in an aberrant γ-chain with 421 residues and γ'-chain with 417 residues.

Patient and Methods

The patient with Ota I was a 37-year-old pregnant woman admitted to hospital due to the

worsening of hypertension. At 34 weeks of gestation, she gave birth (first son) by cesarean section with moderate bleeding and required a blood transfusion. Her and her son's coagulation screening tests revealed low levels of plasma fibrinogen; however, they had no history of other bleeding or thrombotic tendencies. This study was approved by the Ethical Review Board of Shinshu University School of Medicine (#383). After informed consent had been obtained from the patient, a blood sample was collected and coagulation tests, a DNA sequence analysis, and Western blot analysis were performed as described elsewhere [5].

The prothrombin time (PT) (Thromborel S; Sysmex Corporation, Kobe, Japan), activated partial thromboplastin time (APTT) (Thrombocheck APTT-SLA; Sysmex Corporation), and fibrinogen concentrations using the thrombin time method (Thrombocheck Fib-L; Sysmex Corporation), were measured by the automated analyzer, Coapresta 2000 (Sekisui Medical CO., Tokyo, Japan). Immunological fibrinogen concentrations were measured by a latex photometric immunoassay using anti-fibrinogen antibody-coated latex particles (LSI Medicine Co., Tokyo, Japan).

In order to elucidate the mechanisms underlying hypofibrinogenemia, we established seven types of recombinant fibrinogens with FS mutations in the γ- and γ'-chain C-terminal regions produced by Chinese Hamster Ovary (CHO) cell-lines. Fibrinogen concentrations in the culture media and cell lysates of fibrinogen-synthesizing cell lines were measured using an enzyme-linked immunosorbent assay (ELISA). In order to confirm the synthesis of the

variant γ- or γ'-chain in transfected cells, cell lysates were analyzed by sodium dodecyl sulfate and polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting under reducing conditions.

Results

The prothrombin time (PT) and activated partial thromboplastin time (APTT) in Ota I patient were 13.3 seconds (normal range: 10.8 to 13.2) and 38.6 seconds (normal range: 23.0 to 38.0), respectively. The plasma fibrinogen concentration in Ota I patient, measured by the thrombin time method, was 0.68 g/L, while that assessed by the immunological method was 0.64 g/L (normal range: 1.80 to 3.50 g/L). The results of coagulation screening tests performed on the son of Ota I patient when he was 4 months old were as follows: PT: 15.9 seconds, APTT: 36.3 seconds, fibrinogen concentration (the thrombin time method): 0.48 g/L, and fibrinogen concentration (the immunological method.): 0.58 g/L.

A sequence analysis of polymerase chain reaction (PCR)-amplified products from Ota I patient and her son and subcloned PCR-amplified products revealed a heterozygous deletion of AT in *FGG* exon 9; c.1169_1170 del AT (NCBI NM_000509.4) (data not shown), resulting in a FS mutation starting from codon 364 and ending at codon 422, namely, a 48-amino acid substitution and 10-amino acid addition (Table 1). Furthermore, this FS mutation may have influenced the alternative splicing product, the γ'-chain. Therefore, we speculated the

generation of a variant γ’-chain constituted by a 54-amino acid substitution and 10-amino acid truncation, namely, a FS mutation starting from codon 364 and ending at codon 418 (premature termination) (Table 1). We performed a Western blot analysis on the plasma fibrinogen of Ota I patient. No aberrant high-molecular-weight γ- and γ’-chains were observed in her plasma (Fig. 1A and B).

The results of ELISA for seven variant fibrinogen- and two normal fibrinogen-expressing CHO cell-lines established are shown in Figures 1C, 1D, and 1E. The fibrinogen concentration ratio of the medium/cell lysate was 1.57 ± 0.63. Fibrinogen was not detected in the culture media or cell lysates of not only the γ364FS-CHO cell-line, but also the γ370FS-, γ379FS-, and γ385FS-CHO cell-lines. Fibrinogen was also not detected in the culture media or cell lysates of the γ'364FS-CHO cell-line. On the other hand, fibrinogen was synthesized and secreted into the media of the γ387FS- and γ391FS-CHO cell-lines. SDS-PAGE and immunoblotting revealed that all cell lines synthesized variant γ- or γ'-chains as well as Aα- and Bβ-chains, as shown in Figures 1F and 1G. These results demonstrated the absence of the aberrant γ- and or γ’-chain in plasma from Ota I patient, leading to hypofibrinogenemia, and the substituted residues of γLys385-Ile386 for Asn–Asn or Asp–Asn resulted in the loss of the assembly and secretion of both fibrinogens constituting the aberrant γ- or γ'-chain.

Discussion

We previously reported that truncation mutants of the C terminus of the γ-chain shorter than γIle387 demonstrated not only the secretion of aberrant fibrinogens into media, but also their assembly inside cells [6]. We also showed that the variant fibrinogen, γAsp387 indicated marked reductions in the assembly and secretion of aberrant fibrinogen, whereas the variant fibrinogens γArg387, γLeu387, γMet387, and γAla387 did not [7].

Since the crystal structure of the γ-module of the D region (γ143-392) revealed that γIeu387 lies within a β strand composed of residues γ381-387 and also that this strand is constructed by a five-stranded antiparallel β-sheet structure formed among other β strands of γ189-197, γ243-252, γ256-263, and γ275-284 [8, 9], the loss of or alterations in γIeu387 change the tertiary structure of the γ-module and induce defective fibrinogen assembly and secretion. Collectively, these findings and the present results indicate that the γLys385, γIle386, and γIle387 residues and/or conformation of the γ-chain C-terminal tail after 385, but not the length of the γ-chain C-terminal tail, are critical for fibrinogen assembly and secretion [6]. Since the C-terminal end of the γ-chain is flexible, an x-ray diffraction analysis has not provided information on the tertiary structure of γ393-411 [8, 9].

In conclusion, the results of the present study demonstrate that a FS mutation starting from codon 364 and ending at codon 422 for the γ-chain (58 aberrant residues), and premature termination at codon 418 for the γ'-chain (54 aberrant residues), namely Ota I, led to the absence of plasma variant fibrinogen. Expression experiments on five other recombinant FS

mutations in the γ- or γ'-chain C-terminal region demonstrated that the substitution of γLys385-Ile386 led to the loss of the assembly and secretion of both aberrant fibrinogens, and suggest that the γLys385 and γIle386 residues and/or conformation of the γ-chain C-terminal tail are critical for fibrinogen assembly and secretion.

Conflict of Interest Statement

The authors state that they have no conflicts of interest.

Acknowledgments

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Figure legend

Figure 1. Western blot analysis of plasma fibrinogen (A and B).

A healthy volunteer's plasma (NC) and the patient's plasma (Ota I), equivalent to 15 ng (A) and 40 ng (B) fibrinogen, were separated on 8% SDS-PAGE under non-reducing conditions (A) or 10% SDS-PAGE under reducing conditions (B). Blots were developed with an anti-fibrinogen polyclonal antibody. The bands are indicated as Fbg (fibrinogen), Aα, Bβ, γ, and γ-γ.

Synthesis and secretion of variant fibrinogen in transfected CHO cell-lines (C, D, and E). Fibrinogen concentrations in culture media (C) and cell lysates (D) were measured by ELISA. The ratios of the values of the culture medium to the cell lysate are shown in panel E. Mean values are presented with standard deviations indicated by error bars. The values of the variant were significantly different from γN or γ'N (*: $p < 0.05$). γN: wild-type (n=9), γ364FS: γ364 frameshift (n=10), γ370FS (n=10), γ379FS (n=10), γ385FS (n=10), γ387FS (n=10), γ391FS (n=10), γ'N: γ' normal (n=8), and γ'364FS (n=10).

Synthesis of the variant γ- or γ'-chain in transfected CHO cell-lines (F and G). A Western blot analysis of cell lysates. Samples from cell lysates from each selected clone were subjected to 10% SDS-PAGE under reducing conditions. The blots were incubated with an anti-fibrinogen antibody. Lane 1: purified plasma fibrinogen, lane 2: wild-type (γN), lane 3: γ364FS, lane 4: γ370FS, lane 5: γ379FS, lane 6: γ385FS, lane 7: γ387FS, lane 8: γ391FS,

lane 9: purified plasma fibrinogen, lane 10: wild-type γ'N, and lane 11: γ'364FS. The left sides of panels F and G show molecular size markers. The bands are indicated as Aα, Bβ, γ, γ- γ, γ', and γ'364FS.

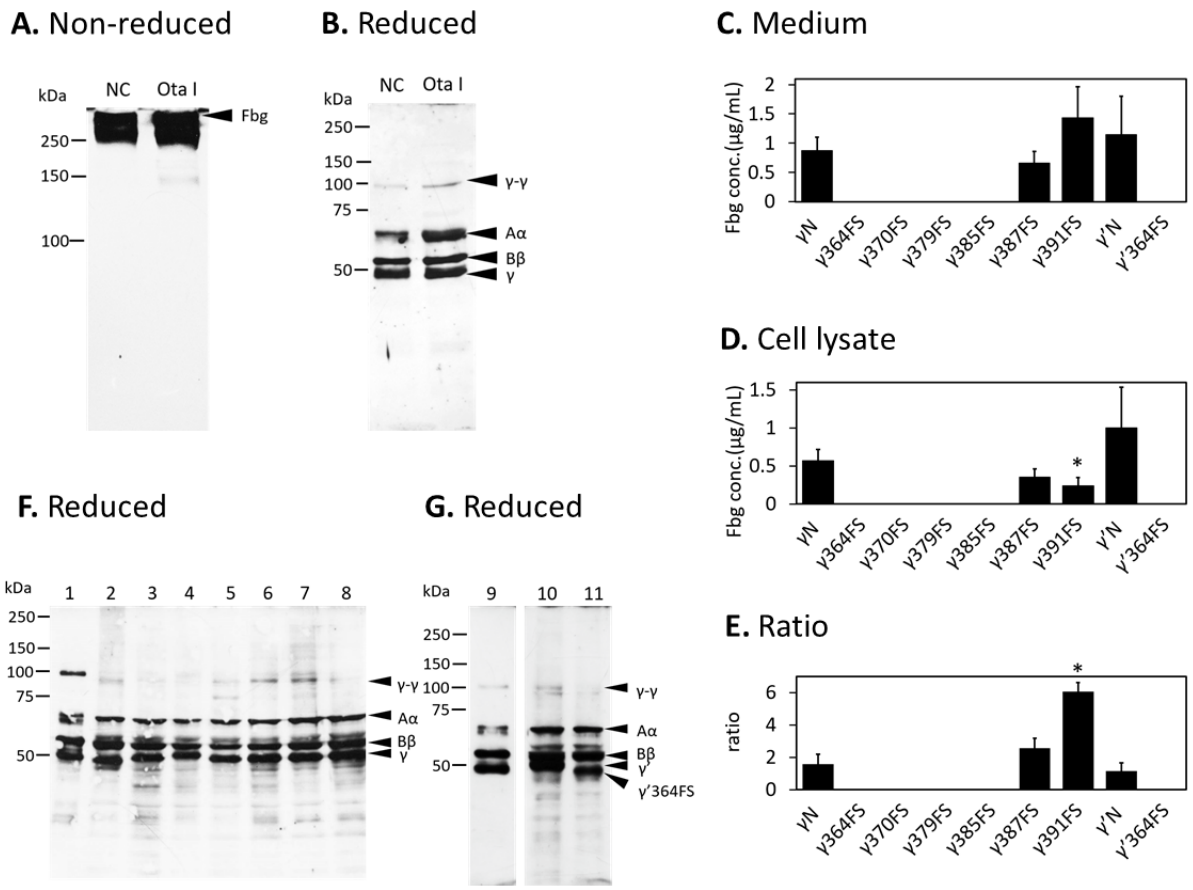

Table 1. Amino acid sequences of patient and recombinant fibrinogen γ- and γ'-chains.

Wild-type and Variants	Amino acid sequences
Number of residues	364 371 381 391 401 411 421
	↓ ↓ ↓ ↓ ↓ ↓ ↓
γN (wild type)	DNGIIWA TWKTRWYSMK KTTMKIIPFN RLTIGEGQQH HLGGAKQAGD V(411)
γ364FS(Ota I)	**EWHYLGH LENPVVFHEE NHYEDNPIQQ THNWRRTATP PGGSQTGWRR LKDRFKRDLL F**(421)
γ370FS	DNGIIWD LENPVVFHEE NHYEDNPIQQ THNWRRTATP PGGSQTGWRR LKDRFKRDLL F(421)
γ379FS	DNGIIWA TWKTRWYSMK NHYEDNPIQQ THNWRRTATP PGGSQTGWRR LKDRFKRDLL F(421)
γ385FS	DNGIIWA TWKTRWYSMK KTTMNNPIQQ THNWRRTATP PGGSQTGWRR LKDRFKRDLL F(421)
γ387FS	DNGIIWA TWKTRWYSMK KTTMKITIQQ THNWRRTATP PGGSQTGWRR LKDRFKRDLL F(421)
γ391FS	DNGIIWA TWKTRWYSMK KTTMKIIPFN THNWRRTATP PGGSQTGWRR LKDRFKRDLL F(421)
γ'N (wild type)	DNGIIWA TWKTRWYSMK KTTMKIIPFN RLTIGEGQQH HLGGAKQVRP EHPAETEYDS LYPEDDL (427)
γ'364FS(Ota I)	**EWHYLGH LENPVVFHEE NHYEDNPIQQ THNWRRTATP PGGSQTGQTR APCGNRI**(417)

Wild-type γ- and γ'-chains and recombinant variant γ- and γ'-chains are indicated (Letters with a bold underline indicate the alternative splicing product called the γ'-chain). Bold letters indicate the amino acid sequences of aberrant γ- and γ'-chains in the fibrinogen of Ota I patient. Letters with a single underline are substituted or added amino acids. The numbers in parentheses indicate all amino acid residues. The second line indicates the number of γ-chain and γ'-chain residues.