Novel variant fibrinogen γp.C352R produced hypodysfibrinogenemia leading to a bleeding episode and failure of infertility treatment

Masahiro Yoda[a], Takahiro Kaido[a], Tomu Kamijo[b, c], Chiaki Taira[a], Yumiko Higuchi[a], Shinpei Arai[a], Nobuo Okumura[a, d]

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

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Address correspondence to:
Shinpei Arai, Ph.D.
Department of Clinical Laboratory Investigation, Graduate School of Medicine,
Shinshu University.
3-1-1 Asahi, Matsumoto, 390-8621 Japan
TEL: +81-263-37-2387
FAX: +81-263-37-2370
e-mail: arais@shinshu-u.ac.jp

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Abstract

Introduction: We identified a patient with a novel heterozygous variant fibrinogen, γp.C352R (Niigata II; N-II), who had a bleeding episode and failed infertility treatment and was suspected to have hypodysfibrinogenemia based on low and discordant fibrinogen levels (functional assay: 0.33 g/L, immunological assay: 0.91 g/L). We analyzed the mechanism of this rare phenotype of a congenital fibrinogen disorder.

Materials and methods: Patient plasma fibrinogen was purified and protein characterization and thrombin-catalyzed fibrin polymerization performed. Recombinant fibrinogen-producing Chinese hamster ovary (CHO) cells were established and the assembly and secretion of variant fibrinogen analyzed by ELISA and western blotting.

Results: Purified N-II plasma fibrinogen had a small lower molecular weight band below the normal γ-chain and slightly reduced fibrin polymerization. A limited proportion of p.C352R fibrinogen was secreted into the culture medium of established CHO cell lines, but the γ-chain of p.C352R was synthesized and variant fibrinogen was assembled inside the cells.

Conclusion: We demonstrated that fibrinogen N-II, γp.C352R, was associated with markedly reduced secretion of variant fibrinogen from CHO cells, that fibrin polymerization of purified plasma fibrinogen was only slightly affected, and that fibrinogen N-II produces hypodysfibrinogenemia in plasma.

Key words: congenital fibrinogen disorders; hypodysfibrinogenemia; fibrinogen γ chain; bleeding; failure of infertility treatment

Introduction

Fibrinogen is a 340 kDa plasma hexametric glycoprotein composed of two sets of three different polypeptide chains Aα, Bβ, γ, which are stabilized by 29 disulfide bonds [1–4]. Three polypeptide chains assembled into a three-chain monomer (Aα-Bβ-γ), are further held together as a six-chain dimer $(Aα\text{-}Bβ\text{-}γ)_2$ in hepatocytes, then finally secreted into the blood and is present at 1.80–3.50 g/L in plasma [5]. The Aα-, Bβ-, γ-chains are encoded by *FGA*, *FGB*, and *FGG*, respectively, and genetic alterations occurring within these genes have been associated with congenital fibrinogen disorders (CFDs) [6]. CFDs can be classified into four different types according to the amounts of functional and antigenic fibrinogen. Afibrinogenemia or hypofibrinogenemia has absent or low plasma fibrinogen antigen levels, and dysfibrinogenemia shows reduced functional activity. Hypodysfibrinogenemia [7] shows reduced antigen levels associated with disproportionately low functional activity.

The carboxyl-terminal region of the fibrinogen γ-chain (γD region) is involved in a variety of functions, not only the primary polymerization hole 'a', high affinity calcium-binding site, and γ-γ crosslinking site [4], but also a tissue-type plasminogen activator (t-PA) binding region for fibrinolysis [8]. Additionally, the γ-chain was stabilized by two intrachain disulfide bonds: Cys^{179}–Cys^{208} and Cys^{352}–Cys^{365} [1]. In addition, several

observations indicated that almost all of fibrinogen storage diseases (FSDs) [9,10] were reported in the γD region. The phenotypes of fibrinogen mutations in γD region are various (hypo-, dys-, and hypodysfibrinogenemia), and also the clinical manifestation is associated with bleeding and thrombosis and more with hereditary hypofibrinogenemia with intrahepatic FSD and no clinical symptoms.

Case reports indicated that γp.C352 variants (γp.C352S, C352Y) caused dys- or hypodysfibrinogenemia according to the fibrinogen variants database [11]. Our previous fibrinogen expression study using Chinese hamster ovary (CHO) cells [12] demonstrated that these variants allowed chain assembly to occur, and the assembled chains were secreted slightly. Namely, there were high concentrations inside the cells and low concentrations in the culture media, suggesting hypofibrinogenemic features and fibrinogen accumulation in CHO cells.

We recently identified fibrinogen Niigata II, a novel heterozygous mutation *FGG* c.1054T>C resulting in the amino acid substitution of γp.C352 with Arg and the disruption of the Cys^{352}–Cys^{365} disulfide bond. In order to clarify the mutational effect on fibrinogen function, accumulation, and secretion, we performed thrombin-catalyzed fibrin polymerization using purified plasma fibrinogen and established recombinant fibrinogen-synthesizing cell lines. Furthermore, we analyzed how these variant

fibrinogens accumulate and localize in CHO cells by immunofluorescence staining. In this report, we have used the native protein nomenclature (add 26 amino acid to mature protein nomenclature for the γ-chain).

Materials and methods

This study was approved by the Ethical Review Board of Shinshu University School of Medicine (#603). After informed consent had been obtained from the patient, blood samples were collected for biochemical and genetic analyses.

Patient and coagulation tests

The proposita of Niigata II was a 38-year-old woman who had an experience of ovarian hemorrhage and received infertility treatment. Her laboratory data showed a markedly lower fibrinogen concentration than the reference interval and she suffered failure of both artificial fertilization and blastocyst. She tried two blastocyst implantations; however, she did not get pregnant. Finally, she successfully became pregnant by supplying fibrinogen preparation before implantation of a blastocyst.

The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentrations, which were measured using the Clauss method, were

evaluated using an automated analyzer, Coapresta 2000 (Sekisui Medical CO., Tokyo Japan). The immunological fibrinogen level was determined using anti-fibrinogen antibody-coated latex particles (Q-May Co., Ohita, Japan).

DNA sequence

Genomic DNA was extracted from whole blood cells using a DNA Extraction WB kit (FUJIFILM-Wako Pure Chemical Ltd., Osaka, Japan) in accordance with the manufacturer's instructions. To analyze all exons and exon-intron boundaries in the Aα-, Bβ-, and γ-chain genes, long-range PCR for *FGA*, *FGB*, and *FGG* was performed using TaKaRa LA Taq (TaKaRa Bio Inc., Otsu Japan) and the three pairs of primers, as described previously [13]. PCR products were purified from agarose gels using a Gene Clean II Kit (Funakoshi, Tokyo, Japan) and sequenced directly using a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit and 3500 Genetic Analyzer (both from Applied Biosystems, Forster City, CA).

Purification of fibrinogen and thrombin-catalyzed fibrin polymerization

The purification of fibrinogen from the patient was performed by immunoaffinity chromatography using an anti-IF-1 monoclonal antibody (LSI Medience) conjugated to

a Sepharose 4B column (IF-1 MoAb Sepharose column) as described previously [14]. The purity and characterization of purified fibrinogen was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in non-reducing conditions (5% polyacrylamide gel) or reducing conditions (10% polyacrylamide gel) and stained with Coomassie Brilliant Blue R-250.

Polymerization was followed by measuring the change in turbidity at 350 nm using a UV-1280 (Shimadzu, Tokyo, Japan). Reactions were performed in a final volume of 100 μL, as described previously [14]. Briefly, fibrinogen (90 μL at 0.20 mg/mL) in 20 mM N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES) was mixed with human α-thrombin (Enzyme Research Laboratories, South Bend, MA, USA, 10 μL at 0.5 U/mL). Three parameters: lag time, maximum slop (Max-slope), and absorbance change in 30 min (ΔAbs), were obtained from the turbidity curves, as described previously [15]. Reactions were performed in triplicate for each sample.

Expression of recombinant fibrinogen

Recombinant variant fibrinogens were prepared as described previously [16,17]. Briefly, the variant γ-fibrinogen expression vector was altered from the pMLP-γ plasmid, which contained wild-type γ cDNA, by oligonucleotide-directed mutagenesis using a

QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) [18] and the following primer pairs (the altered base is underlined); sense 5'-G TTT GAA GGC AAC CGT GCT GAA CAG GAT GGA TC-3' and anti-sense 5'-GA TCC ATC CTG TTC AGC ACG GTT GCC TTC AAA C-3' for γp.C352R , sense 5'-G TTT GAA GGC AAC TTT GCT GAA CAG GAT GGA TCT GG-3' and anti-sense 5'-CC AGA TCC ATC CTG TTC AGC AAA GTT GCC TTC AAA C-3' for γp.C352F in accordance with the instructions. Although γp.C352F has been reported, a variant fibrinogen expression experiment has not been performed. Therefore, we also established γp.C352F fibrinogen-synthesizing CHO cell lines.

The resultant variant and normal expression vectors were co-transfected using a histidinol selection plasmid (pMSVhis) into CHO cell lines that expressed normal human fibrinogen Aα- and Bβ-chains (AαBβ CHO cell lines) using a standard calcium-phosphate coprecipitation method [19]. The cell lines were designated as γp.C352R, γp.C352F, and wild-type CHO cells, respectively. Cells were cultured and colonies were selected on histidinol (Aldrich Chem. Co. Milwaukee, WI, USA), as described previously [13].

Enzyme-linked immunosorbent assay and western blotting analysis

Fibrinogen concentrations in the cell lysates or culture media from the selected clones were measured using an enzyme-linked immunosorbent assay (ELISA) as described previously [20].

Cell lysates of wild-type and variant fibrinogen-synthesizing CHO cells were utilized for western blotting analyses. SDS-PAGE and immunoblotting analysis were performed as described previously [21]. Briefly, blots were developed with a rabbit anti-human fibrinogen polyclonal antibody (DAKO, Glostrup, Denmark) and a mouse anti-human fibrinogen γ chain monoclonal antibody (2G10; Accurate Chemical and Scientific, Westbury, NY, USA). The detection of bands was performed using a ChemiDoc XRS Plus (BIO-RAD, CA, USA).

Immunofluorescence staining

For immunofluorescence microscopy, the cells grown for 4 days on cover-glasses in 60-mm culture dishes were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Localization of intracellular fibrinogen was identified by incubating for 1 h with 1:50 diluted FITC-labeled sheep polyclonal anti-fibrinogen antibody (The Binding Site Group Ltd., Birmingham, UK). Fluorescence was observed using a fluorescence microscope (BX 60; OLYMPUS, Tokyo, Japan). The percentages of

aberrant inclusion body-containing cells were evaluated by counting 200 (wild type, γp.C352R, γp.C352F) or 400 (γp.R401W, γp.C352S, γp.C352Y) cells for each cell line.

Statistical analysis

The Kruskal–Walls test and the Steel–Dwass test were used to compare recombinant fibrinogen production using EZR software [22]. Box-and-whisker plots and dot plots were prepared using the R software (version 4.0.3). Three parameters of fibrin polymerization were analyzed using Welch's t-test. Differences were considered significant when the p-value was <0.05.

Results

Coagulation tests and DNA sequence

PT and APTT in the patient were 13.1 sec (normal range: 10.0–13.0 sec) and 29.8 sec (normal range: 23.0–38.0 sec), respectively. Her plasma fibrinogen concentration, measured using the Clauss method, was 0.33 g/L (normal range: 1.80–3.50 g/L), and that assessed by the immunological method was 0.91 g/L (normal range: 1.80–3.50 g/L).

Sequence analysis of PCR-amplified products revealed a heterozygous T>C single nucleotide mutation at position 1054 in *FGG* exon 8 (c.1054T>C; NCBI NG_008834.1)

resulting in the substitution of Cys (TGT) for Arg (CGT) at γp.352 residue (native protein) (γp.C352R) (data not shown).

Characterization of plasma fibrinogen and thrombin-catalyzed fibrin polymerization

The characterization of purified plasma fibrinogen was determined by SDS-PAGE. In non-reducing conditions, all variant fibrinogens showed typical patterns. Niigata II fibrinogen showed the same pattern as normal fibrinogen (Figure 1A), whereas, in reducing conditions Niigata II fibrinogen showed small amounts of an additional lower molecular weight band below the normal γ-chain and increases in degradation bands derived from the Aα-chain (Figure 1B). These results indicated that amount of affected γ-chain possessing fibrinogen was markedly reduced compared with that of normal γ-chain possessing fibrinogen in Niigata II plasma fibrinogen.

Thrombin-catalyzed fibrin polymerization was monitored as the change in turbidity at 350 nm. Representative turbidity curves are shown in Figure 2. In conditions with 0.18 mg/mL fibrinogen in the presence of 1.0 mM $CaCl_2$, polymerization of Niigata II plasma fibrinogen was reduced compared with that of normal fibrinogen. Although the differences in lag time and Max-slope between normal plasma fibrinogen and the Niigata II plasma fibrinogen were not significant, Niigata II was a significantly lower endpoint

of turbidity (0.233 ± 0.012) compared with a normal plasma fibrinogen (0.331 ± 0.006) (p<0.01).

Synthesis and secretion of recombinant variant fibrinogens in CHO cells

We established wild-type (WT), γp.C352R and γp.C352F fibrinogen-synthesizing CHO cells. Fibrinogen concentrations in the culture media and cell lysates of fibrinogen-synthesizing cell lines are shown in Figure 3. The median (and interquartile range) of WT (n = 9) fibrinogen concentrations were 0.646 (0.529–0.822) μg/mL in the culture media and 0.653 (0.452–0.732) μg/mL in cell lysates, resulting in a ratio of culture media to cell lysates (ratio) of 1.207 (1.171–1.346). As for variant fibrinogen-synthesizing CHO cells, the fibrinogen concentrations in the culture media of γp.C352R (n = 11) and γp.C352F (n = 10) were 0.135 (0.092–0.150) and 0.101 (0.073–0.153) μg/mL respectively. They were both significantly lower than that of WT. In the cell lysates, the fibrinogen concentrations of γp.C352R and γp.C352F were 4.385 (3.216–5.642) and 5.436 (4.569–6.204) μg/mL respectively. They were both significantly higher than that of WT. Thus, the calculated ratio of the variant fibrinogen-synthesizing CHO cells was significantly lower than that of WT [γp.C352R: 0.030 (0.020-0.036), γp.C352F: 0.024 (0.015-0.030)]. There were no significant differences in fibrinogen synthesis and secretion between the two variant fibrinogen-synthesizing CHO cells.

To observe the assembly of intact fibrinogen and/or synthesis of a mutant γ-chain, SDS-PAGE and western blotting analysis were performed in non-reducing or reducing conditions. In non-reducing conditions, intact fibrinogen was observed in cell lysates of two fibrinogen variants including a wild-type fibrinogen-synthesizing cell line (Figure 4 A). In reducing conditions, not only the Aα- and Bβ-chain but also γ-chain were observed in cell lysates of two variant fibrinogens and wild-type fibrinogen-synthesizing cell lines (Figure 4 B, C). Of note, the γ-chain bands of two variant fibrinogen had slightly faster mobility than that of wild-type fibrinogen. Namely, γp.C352R and γp.C352F showed 2.2 and 1.6 kDa lower relative molecular weight than wild-type fibrinogen, respectively (Figure 4 C).

Fluorescent fibrinogen staining

To analyze the localization of variant fibrinogen inside the established CHO cells, in addition to γp.C352R and F, γp.C352S and Y (which were established previously [12]), a direct immunofluorescence test with FITC labeled anti-fibrinogen antibody was performed as described in the Materials and Methods. Original CHO cells (Figure 5) did not stain fibrinogen. Wild-type fibrinogen-synthesizing (γp.352C-CHO) cells were stained in a perinuclear network pattern (abbreviated as 'N'). Variant fibrinogen-

synthesizing cell lines, γp.R401W showed two characteristic patterns, namely, scattered large-granular bodies (abbreviated as 'LG'), fibrous forms of two subpolar sites of the nucleus (abbreviated as 'F'), previously reported as a specific feature in fibrinogen variants causing hepatic ER storage disease [23]. Although C352S-CHO cells showed 'N' and 'LG' staining patterns and the frequency of 'LG' was 23.00 %, the other three variant fibrinogen-synthesizing cell lines (γp.C352Y, R and F) were stained with only the 'N' pattern.

Discussion

We identified the novel heterozygous variant fibrinogen, γp.C352R, which was designated as fibrinogen Niigata II. We considered that this substitution caused hypodysfibrinogenemia because the proposita's decreased functional and antigenic fibrinogen levels were 0.33 and 0.91 g/L, respectively, and the functional/antigenic fibrinogen ratio was 0.363 and markedly lower than the reference interval (0.800–1.200). Therefore, we performed thrombin-catalyzed fibrin polymerization using purified plasma fibrinogen and analyzed the production of variant fibrinogen using established CHO cell lines. The results revealed that the fibrin polymerization of fibrinogen Niigata II was slightly reduced due to a small amount of variant fibrinogen, which may also be due to

the increased degradation of Aα-chain observed in purified fibrinogen Niigata II. For the analysis of recombinant fibrinogen production just a limited proportion of p.C352R fibrinogen was secreted into the culture media, although the γ-chain of p.C352R was synthesized, and variant fibrinogen was assembled inside the cells. In other words, the γp.C352R substitution markedly impaired secretion of fibrinogen from CHO cells in vitro.

According to the fibrinogen variant database [11], three species and six families of missense mutations in the γp.352 residue have been reported (Table 1). We have already established and reported two variant fibrinogen-synthesizing CHO cell lines, γp.C352S or γp.C352Y. Although fibrinogen Melbourne (γp.C352F) [29] has been reported to cause hypodysfibrinogenemia, a variant fibrinogen expression experiment has not been performed. Therefore, in addition to the γp.C352R cell line, we established γp.C352F fibrinogen-synthesizing CHO cell lines. The variant cell line, γp.C352F, also synthesized mutant γ-chain, assembled them into fibrinogen inside the cells, and secreted small amounts of variant fibrinogen into the culture media. Furthermore, variant fibrinogen concentrations in the cell lysates were much higher than those in the normal cell line, namely, variant fibrinogen accumulated inside the cell. These results were similar to two other established cell lines (γp.C352S, C352Y) [12]. These data indicated that substitutions of γp.C352 led to a drastic change in the tertiary structure of the γ-module

caused by the destruction of an intra-peptide disulfide bridge, resulting in reduced secretion of variant fibrinogen and impaired function of polymerization. Finally, five out of seven families possessing substitutions of γp.C352, namely, Cordoba [24], Tokai [25], Frostburg [28], Melbourne [29], and Niigata II, showed reduced antigen levels associated with disproportionately low functional levels, namely hypodysfibrinogenemia.

Although the fibrinogen Niigata II proposita experienced a bleeding episode and failure of both artificial fertilization and blastocyst implantation, at least one member of each other family with a γp.C352 substitution manifested thrombotic episodes except fibrinogen Cordoba, which was an extremely low functional fibrinogen level; 0.12 g/L, and manifested bleeding. The crucial step in the fibrinolytic process is the activation of plasminogen to plasmin by tissue-type plasminogen activator (t-PA). Fibrinolysis occurs when t-PA binds to γp.338–350 (312–324, mature protein nomenclature) region [32] on the fibrin surface. In this region, two thrombotic variants, γp.S358C [34] and G359C [35], have been reported. In the latter case, the proximity of the γp.G359C mutation to the Cys^{352}–Cys^{365} disulfide bond may disrupt this disulfide bond [35], resulting in thrombotic manifestation. These reports suggested that amino acid substitutions from or to Cys around γp.352 are prone to arouse thrombotic complications. On the other hand, although γp.C365S cases Pegasus Bay [30] and Italian [31] should cleave the Cys^{352}–Cys^{365}

disulfide bond, their clinical outcomes were both hemorrhages. However, we did not know whether the variant fibrinogens were present in the patient plasma. It is important to clarify whether variant fibrinogens are present in their plasma or not, because there is a possibility that these variant fibrinogens may cause thrombosis. Some inherited thrombophilia cases have already shown to be a risk factor for reproductive disorders including recurrent pregnancy loss [36]. Therefore, this information may help to better predict the clinical manifestation.

Beside the above coagulation or fibrinolytic complications, a notable factor was fibrinogen storage in CHO cells. Neighboring the Cys^{352}–Cys^{365} in the γD region, eight hypofibrinogenemic variants (γp.G310R [37], γp.T340P [38], γp.D342N and p.G392S [39], γp.H366D [40], deletions γp.G372-Q376 [41], γp.T397I [42], γp.G401W [43]) have already been reported as FSD-inducible mutations [44,45]. In a previous report [23], it has been already verified that the construction of variant fibrinogen-synthesizing cells and the immunofluorescence method were able to be utilized in the non-invasive screening of FSD-inducible variant fibrinogen. Briefly, the detection of the 'F' form in variant fibrinogen-synthesizing CHO cells may be specific to the FSD-inducible type of the hypofibrinogenemic variant. Then, we investigated whether the 'F' forms exist in four variant fibrinogen-synthesizing CHO cells (γp.C352S, C352Y, C352R, C352F). We did

not detect the ‘F’ form in any variant fibrinogen-synthesizing CHO cells, namely, substitution variants at γp.C352 possessed a markedly reduced possibility of the onset of FSD.

In conclusion, genetic analyses revealed a novel heterozygous mutation, *FGG* c.1054T>C in exon 8, namely p.γC352R. The variant fibrinogen expression experiment and thrombin-catalyzed fibrin polymerization of patient’s plasma fibrinogen suggested that the variant fibrinogen was slightly present in Niigata II plasma and indicated lower functional activity, resulting in a manifestation of hypodysfibrinogenemia. Following immunofluorescence staining results suggested substitution at γp.352 residue may not be associated with the onset of FSD. Furthermore, case reports suggested that the Cys^{352}–Cys^{365} disulfide bond is important for not only fibrinogen secretion and function but also fibrinolysis.

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Author contributions

Masahiro Yoda, Takahiro Kaido, Tomu Kamijo performed the experiments. Masahiro Yoda wrote the paper. Chiaki Taira, Yumiko Higuchi, Shinpei Arai and Nobuo Okumura designed the research and discussed the data. Shinpei Arai and Nobuo Okumura reviewed the paper.

Compliance with ethical standards

Conflict of interest

The authors state that they have no conflicts of interest.

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Table 1 Clinical data on variant fibrinogens associated with disruption of Cys^{352}–Cys^{365}disulfide bond.

Mutation	Name [References]	Patient and family	Manifestation	Functional fibrinogen level (g/L)	Antigenic fibrinogen level (g/L)	Ratio	Hemorrhage	Thrombosis	Recurrent pregnancy loss
γp.C352S	Cordoba [24]	Propositus	hypodys	0.12	1.42	0.085	Yes	No	No
		Son		0.25	0.81	0.309	No	No	No
	Tokai [25]	Propositus	hypodys	0.31	1.02	0.304	No	Yes	No
	USA 12 [26]	Propositus	dys	0.48	1.48	0.324	No	Yes	No
γp.C352Y	Suhl [27]	Proposita	dys	< 0.6	1.5	< 0.400	No	Yes	No
	Frostburg [28]	Propositus	hypodys	0.5	0.4	1.25	No	Yes	No
		Sister 1		0.7	0.5	1.4	No	No	No
		Sister 2		0.6	0.6	1	No	No	No
γp.C352F	Melbourne [29]	Propositus	hypodys	0.4	0.8	0.5	No	Yes	No
		Father		0.7	1.1	0.636	No	No	No
γp.C352R	Niigata II [this report]	Proposita	hypodys	0.33	0.91	0.363	Yes	No	Yes
γp.C365S	Pegasus Bay [30]	Proposita	hypo	0.6	0.5	1.2	Yes	No	No
	Italian [31]	Propositus	hypodys	0.47	1.25	0.376	Yes	No	No

ND: not described

Figure legends

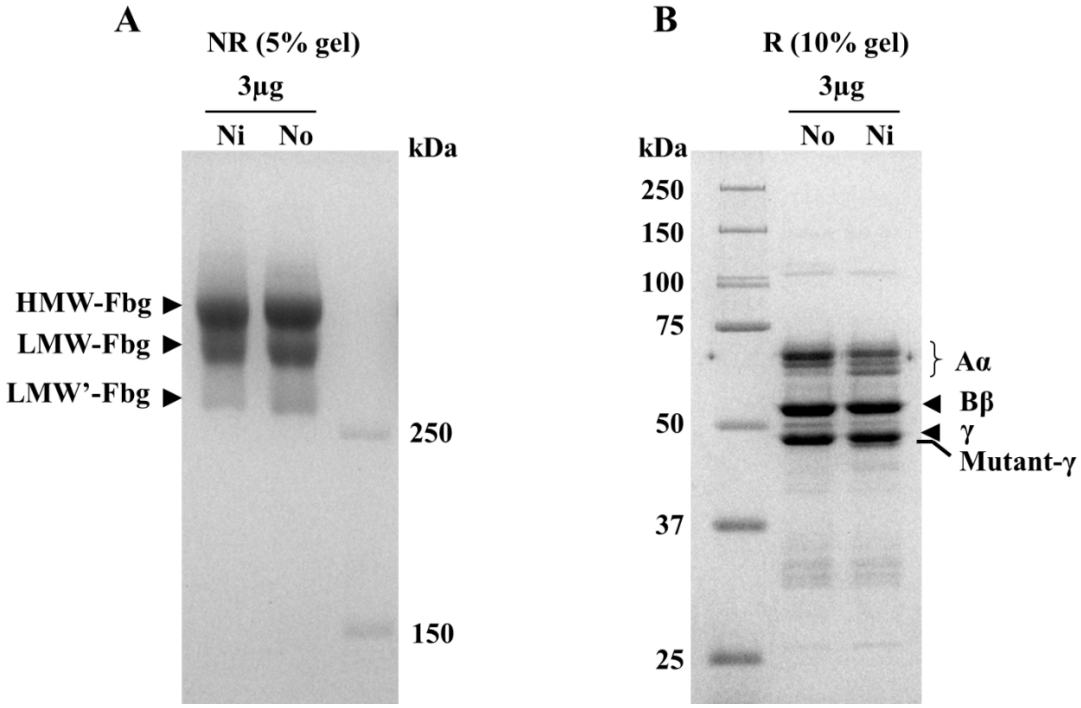


Fig. 1 Characterization of purified plasma fibrinogens

Purified plasma fibrinogens were resolved using SDS-PAGE in non-reducing conditions (NR) (Panel A) or reducing conditions (R) (Panel B). The gels shown in panel A and B (applied 3 μg of fibrinogen) were stained with Coomassie Brilliant Blue R-250. No; normal fibrinogen, Ni; Niigata II fibrinogen. HMW; high-molecular-weight fibrinogen, LMW; low-molecular-weight fibrinogen, LMW'; low-molecular-weight' fibrinogen are indicated in Panel A. The structural difference between LMW-fibrinogen and LMW'-fibrinogen is the removal of a 35 kDa carboxyterminal polypeptide from one or two Aα-chain(s), respectively. Aα, Bβ, γ and mutant-γ chains are indicated in Panel B.

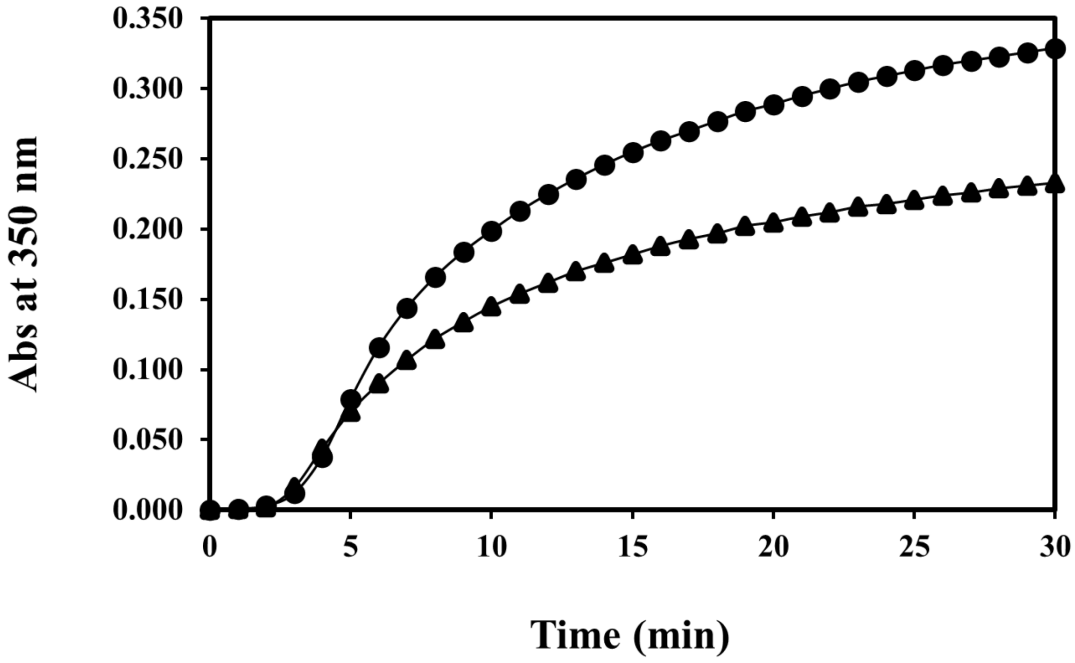


Fig. 2 Thrombin-catalyzed fibrin polymerization

The polymerization of purified plasma fibrinogens (0.18 mg/mL) was initiated with thrombin (0.05 U/mL) and monitored at 350 nm. We conducted experiments in triplicate, and representative polymerization curves are indicated. ●: normal plasma fibrinogen, ▲: Niigata II plasma fibrinogen.

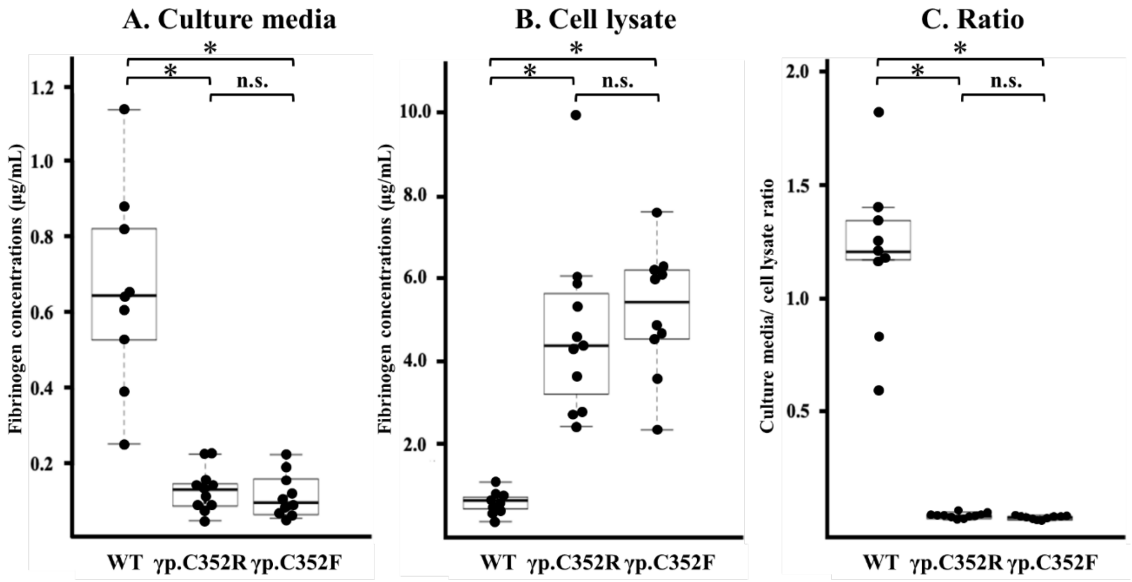


Fig. 3 Synthesis of γp.C352R and C352F fibrinogen in transfected CHO cell lines

Fibrinogen concentrations in culture media (A) and cell lysates (B) were measured using ELISA as described in the Materials and Methods. The ratios of values of the culture media to the cell lysate are shown in (C). Box plots and the central bar show the interquartile range and median, respectively. The whiskers indicate the minimum and maximum value excluding outliers. Dots represent individual values. Concentrations were assessed for clones expressing wild-type (WT, n=9), γp.C352R (n=11), and γp.352F (n=10). The significance of differences among WT and variant fibrinogen-synthesizing CHO cells was assessed using the Kruskal–Walls test and the Steel–Dwass test. *p < 0.01; n.s.: Non-significant.

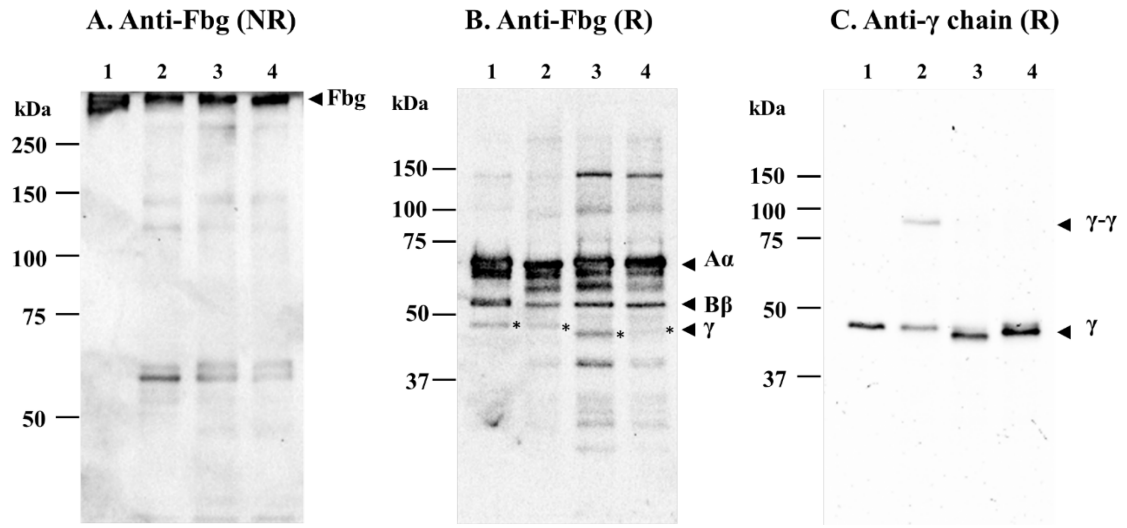
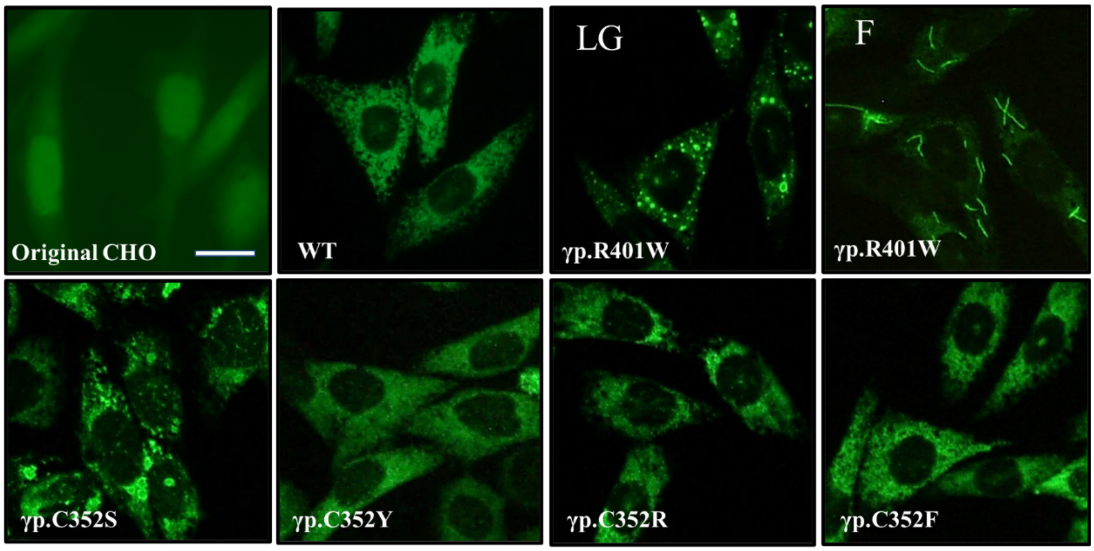


Fig. 4 Western blotting analysis of CHO cell lysates

SDS-PAGE run in non-reducing conditions using 7.5% gels (Panel A) and in reducing conditions using 10% gels (Panels B and C). Blots were developed with an anti-human fibrinogen polyclonal antibody (Panels A and B), and an anti-γ chain monoclonal antibody (Panel C) as described in the Material and Methods. Size markers are shown on the left side of each panel. The bands shown with an asterisk '*' in Panel B indicate γ chain of each sample. 1: Purified recombinant wild-type fibrinogen. 2: Wild-type fibrinogen-synthesizing CHO cell. 3: γp.C352R fibrinogen-synthesizing CHO cell. 4: γp.C352F fibrinogen-synthesizing CHO cell. NR; Non-reducing conditions, R; Reducing conditions.



Inclusion body	WT	γp.R401W	γp.C352S	γp.C352Y	γp.C352R	γp.C352F
LG (%)	0	20.75	23.00	0	0	0
F (%)	0	20.50	0	0	0	0
N (%)	100	58.75	77.00	100	100	100

Fig. 5 Immunofluorescence staining for variant fibrinogen-synthesizing CHO cell lines

We stained original CHO cells as a negative control, wild-type (WT) fibrinogen-synthesizing CHO cells as a normal control (N), 'LG' and 'F' in γp.R401W fibrinogen-synthesizing CHO cells indicate positive for scattered large granular form and fibrous form, respectively. γp.C352S, γp.C352Y, γp.C352R, and γp.C352F indicate variant fibrinogen-synthesizing CHO cells. The percentages of the 'LG', 'F', or normal 'N' form were shown below the photo. Scale Bar: 20 μm.