Congenital fibrinogen disorder with a compound heterozygote possessing two novel *FGB* mutations, one qualitative and the other quantitative

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Key words

hypodysfibrinogenemia, splicing abnormality, *FGB*, nonsense-mediated mRNA decay,

fibrin polymerization

Abbreviations

APTT, activated partial thromboplastin time; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; HEPES, N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]; NMD, nonsense-mediated mRNA decay; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PT, prothrombin time; RT, reverse-transcriptase; SDS, sodium dodecyl sulfate; TCFP, thrombin-catalyzed fibrin polymerization; UTR, untranslated region.

1. Introduction

Fibrinogen is a 340 kDa plasma hexametric glycoprotein composed of two sets of three different polypeptide chains Aα, Bβ, γ, which are mainly synthesized and assembled into a three-chain monomer (Aα-Bβ-γ) by disulfide bonds, further held together as a six-chain dimer $(Aα\text{-}Bβ\text{-}γ)_2$ by disulfide bonds in hepatocytes, then finally secreted into the blood and is present at 1.8–3.5 g/L in plasma [1]. The Aα-, Bβ-, γ-chains are composed of 610, 461, and 411 residues, which are encoded by *FGA*, *FGB*, and *FGG*, respectively, that are clustered in a 50 kb region on the long arm of chromosome 4q31 [2].

Congenital fibrinogen disorders (CFD) are due to genetic alterations occurring within genes coding for *FGA*, *FGB*, and *FGG*, and can be classified into four different manifestations according to the amounts of functional and antigenic fibrinogen. Afibrinogenemia or hypofibrinogenemia has absent or low plasma fibrinogen antigen levels (quantitative fibrinogen deficiencies) and dysfibrinogenemia shows reduced functional activity (qualitative fibrinogen deficiencies). Hypodysfibrinogenemia shows reduced antigen levels associated with disproportionately low functional activity (quantitative and qualitative fibrinogen deficiencies) [3]. In afibrinogenemia and hypofibrinogenemia, mutations in the fibrinogen genes may lead to a deficiency in fibrinogen by several mechanisms: these can act at the DNA level, at the RNA level by

affecting mRNA splicing or stability, or at the protein level by affecting synthesis, assembly, or secretion [4]. On the other hand, dysfibrinogenemia is mostly found in heterozygous carriers of missense variants, and mutations can affect fibrinogen functions at different levels such as fibrin polymerization or fibrinopeptide cleavage [5].

Hypodysfibrinogenemia, which has been reported at a low frequency, shares features with both hypo- and dysfibrinogenemia: the decreased levels of circulating fibrinogen lead to a hypofibrinogenemic phenotype, whereas the dysfunction of fibrinogen molecules is related to the dysfibrinogenemic phenotype, thereby quantitative and qualitative fibrinogen deficiencies can coexist [3]. Several molecular mechanisms leading to hypodysfibrinogenemia have been identified, including heterozygosity for missense or nonsense mutations, or compound heterozygosity for missense and/or nonsense mutations [3]. According to previous hypodysfibrinogenemia reports [6], compound heterozygous cases are so rare that there were only 7 cases among 39 hypodysfibrinogenemia reports. In general, compound heterozygosity cases [6-8] are more unusual.

We recently identified a novel compound heterozygous variant (designed as Kobe I) with *FGB* 35bp c.1245 -17_1262 or -16_1263 del and *FGB* c.510T>A, mutations on different alleles. The former was at the boundary of the *FGB* intron 7-exon 8, and the

latter was *FGB* exon 4. Functional and antigenic fibrinogen values showed reduced antigen levels associated with disproportionately low functional activity, suggesting hypodysfibrinogenemia. In order to clarify the molecular mechanisms leading to hypodysfibrinogenemia in Kobe I, we produced a minigene incorporating the deletion region, transfected them into Chinese hamster ovary (CHO) cell line and analyzed reverse-transcriptase polymerase chain reaction (RT-PCR) products. We also established a recombinant fibrinogen-producing CHO cell line with an *FGB* c. 510T>A mutation. Using this recombinant variant fibrinogen, we analyzed its assembly and secretion.

2. 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.

2.1. Patient and coagulation tests

The proposita of Kobe I was a 31-year-old woman who had continuous moderate bleeding in the uterus several days after first vaginal delivery. Her laboratory data showed a lower fibrinogen concentration than reference interval, but she and her family members

had not experienced any episodes of abnormal bleeding or thrombosis.

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

2.2. Characterization of plasma fibrinogen

Patient plasma fibrinogen was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in reducing conditions (10% polyacrylamide gel) and immunoblotting using a rabbit-anti-human fibrinogen antibody (DAKO, Carpinteria, CA, USA) or rabbit anti-human Bβ-chain antibody (Chemicon International, Temecula, CA, USA) [9]. Reacting species were visualized with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Medical and Biological Laboratories Ltd., Nagoya, Japan) and enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blot were then exposed using a ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA).

115 *2.3. Mutational screening*

116 Genomic DNA was extracted from whole blood cells using a DNA Extraction WB
117 kit (FUJIFILM-Wako Pure Chemical Ltd., Osaka, Japan) in accordance with the
118 manufacturer's instructions. In order to analyze all exons and exon-intron boundaries in
119 the Aα-, Bβ-, and γ-chain genes, 32 PCR primers were designed for DNA amplification
120 by PCR as described previously [10, 11]. PCR products were purified from agarose gels
121 using a Gene Clean II Kit (Funakoshi, Tokyo, Japan) and directly sequenced using a
122 BigDye™ Teminator Cycle Sequencing Ready Reaction Kit and 3500 Genetic Analyzer
123 (both from Applied Biosystems, Forster City, CA). To verify the deletion of the
124 nucleotides or mutation detected by direct sequencing, the PCR products were subcloned
125 into pCR®4-TOPO plasmid vectors (Invitrogen, Carlsbad, CA, USA) in accordance with
126 the manufacturer's instructions, and the extracted subcloned plasmid vectors were
127 sequenced as described above.

128

129 *2.4. Mutation specific PCR*

130 We performed PCR to identify whether the two mutations in *FGB* were on the same
131 allele or not using primer pairs: sense primer located in *FGB*-IVS3 (5'-
132 CTGCTTGGTGATAGCTCAGT-3') and antisense primer located at the boundary of

133 *FGB* intron 7-exon 8, which is specific to the allele with the 35 bp deletion (5'-
134 CTTTAGAACACTGTTTTTGGCG-3'). PCR products were purified from agarose gels
135 and directly sequenced as described above.
136
137 *2.5. Construction of expression vectors for the analysis of fibrinogen Bβ-chain gene*
138 *transcripts*
139 DNA fragments spanning from exon 7 to the 3' untranslated region (UTR) of *FGB*
140 were amplified using the patient's genomic DNA and primer pairs: sense primer located
141 in *FGB*-exon 7 (5'-GGACCCACAGAACTTTTGATAG-3') and antisense primer located
142 in the *FGB*-UTR (5'-GACTTGGAGTGAAGTGTTGAG-3'). The purified PCR products
143 of the minigene (wild-type; 2217 bp, mutant type 2182 bp) were inserted into a plasmid
144 vector (pcDNA 3.1™/V5-His-TOPO®; Invitrogen, Carlsbad, CA, USA) and transfected
145 into competent cells (One Shot® TOP10 chemically competent cells; Invitrogen, Carlsbad,
146 CA, USA) in accordance with the manufacturer's instructions. Plasmid DNA was purified
147 using a QIAprep® Spin Miniprep Kit (QIAGEN N.V., Hulsterweg, The Netherlands) and
148 the nucleotide sequences were confirmed.
149 Expression vectors Bβ-wt and Bβ-mt were introduced into CHO cells using
150 Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA, USA), as described previously

151 [12, 13]. Transfected CHO cell lines were cultured in 5% CO_2 at 37°C and harvested 48
152 h after transfection. Total cellular RNA was extracted from cells using a QIAamp RNA
153 Blood Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's
154 instructions. RT reactions were performed using oligo dT primer and RT from Molony
155 murine leukemia virus [13], followed by PCR amplification with a pair of primers: sense
156 primer located *FGB*-exon 7 (same primer as for minigene preparation) and antisense
157 primer located in the *FGB*-UTR (5'-GAACGCTTCTCCTTCCTTAC-3'). RT-PCR-
158 amplified products were separated by electrophoresis on 1% agarose gels and purified
159 from the gels. DNA fragments were sequenced as described above using the primers used
160 for RT-PCR and others [10, 11].
161
162 *2.6. Expression of recombinant fibrinogen*
163 Recombinant variant fibrinogens were prepared as described previously [14, 15].
164 Briefly, the variant Bβ-fibrinogen expression vector pMLP-Bβ510T>A was altered from
165 the pMLP-Bβ plasmid (kindly provided by Lord ST, University of North Carolina, Chapel
166 Hill, NC, USA), which contained wild-type Bβ cDNA, by oligonucleotide-directed
167 mutagenesis using a Quick change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla,
168 CA, USA) [16] and the following primer pair (altered base is underlined); sense: 5'-

169 GTAGTCAAAGAGTACTCCTCAGAACTGGAAAAGCACC-3' and antisense: 5'-
170 GGTGCTTTTCCAGTTCTGAGGAGTACTCTTTGACTAC-3' for Bβ510T>A.

171 The resultant variant and normal expression vectors were co-transfected using a
172 histidinol selection plasmid (pMSVhis) into CHO cell lines that expressed normal human
173 fibrinogen Aα- and γ-chains (Aαγ CHO cell lines) using a standard calcium-phosphate
174 coprecipitation method [17]. The cell lines were designated as Bβ510T>A- and normal
175 Bβ-CHO cells, respectively. Cells were cultured and colonies were selected on histidinol
176 (Aldrich Chem. Co. Milwaukee, WI, USA), as described previously [18]. Fibrinogen
177 concentrations in the cell lysates or culture media from the selected clones were measured
178 using an enzyme-linked immunosorbent assay (ELISA) [19].

179

180 *2.7. Purification of plasma fibrinogen*

181 The purification of fibrinogen from the patient was performed by immunoaffinity
182 chromatography using an anti-IF-1 monoclonal antibody (LSI Medience) conjugated to
183 a Sepharose 4B column (IF-1 MoAb Sepharose column) [20]. The fibrinogen solution
184 was loaded onto the IF-1 MoAb Sepharose column equilibrate with 20 mmol/L Tris-HCl,
185 pH 7.4, 0.3 mol/L NaCl, 1 mmol/L $CaCl_2$. The column was washed sequentially with 20
186 mmol/L Tris-HCl, pH 7.4, 1 mol/L NaCl, 1 mmol/L $CaCl_2$ and 50 mmol/L sodium acetate,

187 pH 6.0, 0.3 mol/L NaCl, 1 mmol/L $CaCl_2$. Fibrinogen was eluted with 20 mmol/L Tris-
188 HCl, pH 7.4, 0.3 mol/L NaCl, 12.5 mmol/L EDTA, and dialyzed against 20 mmol/L N-
189 [2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH7.4, 0.12 mol/L
190 NaCl, 2 mmol/L $CaCl_2$ at 4℃ and purified fibrinogen concentrations were measured as
191 described previously [21].
192
193 *2.8. Thrombin-catalyzed fibrin polymerization*
194 The turbidity curves of fibrin polymerization were recorded at 350 nm using a UV-
195 1280 (Shimadzu, Tokyo, Japan). Human α-thrombin (Enzyme Research Laboratories,
196 South Bend, MA, USA)-catalyzed fibrin polymerization (TCFP) was performed, as
197 described previously [21]. Briefly, fibrinogen (90 μL at 0.20 mg/mL) in 20 mM HEPES
198 was mixed with human α-thrombin (10 μL at 0.5 U/mL). Three parameters: lag time,
199 maximum slop (Max-slope), and absorbance change in 30 minutes (ΔAbs), were obtained
200 from the turbidity curves, as described previously [11]. Reactions were performed in
201 triplicate for each sample.
202
203 *2.9. Statistical analysis*
204 The significance of differences in fibrinogen production between variant Bβ- and

normal Bβ-CHO cell lines, and three parameters of fibrin polymerization were analyzed using a one-way Mann–Whitney U test and Welch's t test, respectively. A difference was considered to be significant when the p-value was < 0.05.

3. Results

3.1. Coagulation screening tests and DNA sequence analysis

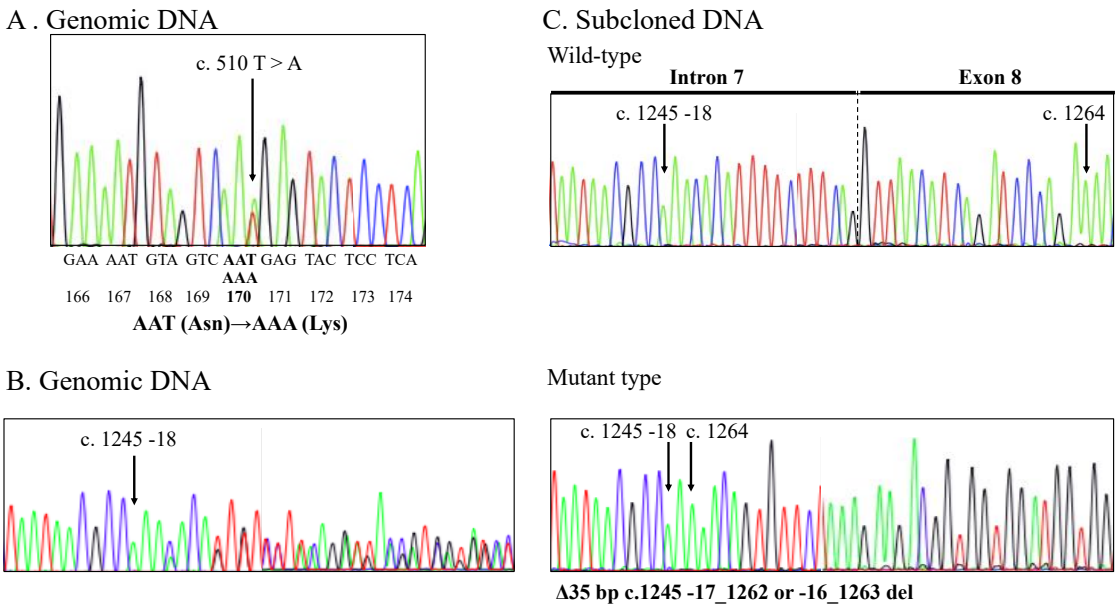
PT and APTT in the Kobe I patient were 12.6 (normal range: 10.0–13.0 s) and 29.0 (normal range: 23.0–38.0 s), respectively. Her plasma fibrinogen concentration, measured using the Clauss method, was 1.05 g/L, and that assessed by the immunological method was 1.24 g/L (normal range: 1.80–3.50 g/L).

Sequence analysis of PCR-amplified products and subcloned PCR-amplified products revealed a heterozygous T>A single nucleotide mutation at position 510 in *FGB* exon 4 (c. 510T>A; NCBI NG_008833) (Figure 1, A) resulting in the substitution of Asn (AAT) for Lys (AAA) at Bβ170 residue (native protein) (Bβp.N170K). Furthermore, analysis showed a compound heterozygous deletion at the boundary of *FGB* intron 7-exon 8 (Figure 1, B. Genomic DNA). Additional PCR amplification was performed using *FGB*-IVS7 and *FGB*-UTR primers, and the product (wild-type; 439 bp) was subcloned into the pCR®4-TOPO plasmid vectors and sequenced. Sequence results revealed the

223 presence of two clone species with 35 bp deletion negative (wild-type) and 35 bp c.1245
224 -17_1262 or -16_1263 del positive (mutant type, called the 35 bp deletion) (Figure 1, C.
225 Subcloned DNA).

226 We performed PCR and DNA sequence using primer pairs specific to the 35 bp
227 deletion. As a result, there was no mutation at *FGB* c.510 (not shown in the figure). In
228 brief, these two mutations (c. 510T>A, the 35 bp deletion) were on the different alleles.

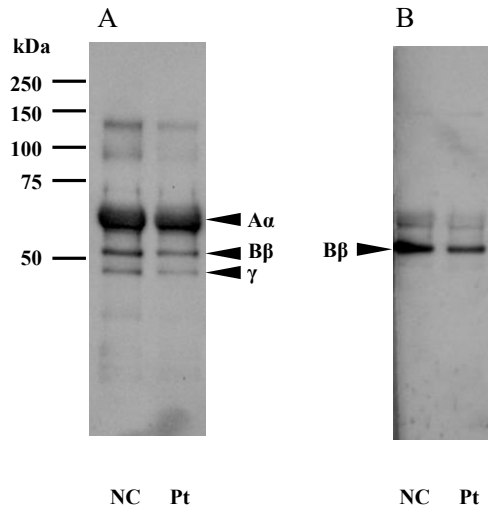
229



230 **Double column, color**

231 **Figure 1. Nucleotide sequences of fibrinogen Bβ-chain genes in Kobe I.** The PCR-
232 amplified Bβ-chain genes of genomic DNA (A, B) and subcloned DNA (C) in the Kobe
233 I patient were sequenced using forward primers. A and B indicate exon 4 and the boundary

234 of intron 7-exon 8, respectively. The boundary of intron 7-exon 8 of subcloned genes
235 (wild-type and mutant type) was sequenced, respectively (C). Sequence results indicate
236 FGB c. 510 T>A (A) and 35 bp c.1245 -17_1262 or -16_1263 del (C).
237
238 *3.2. Characterization of plasma fibrinogen*
239 We performed a western blotting analysis on Kobe I plasma fibrinogen. In reducing
240 conditions, three bands compatible with the normal Aα-, Bβ-, and γ-chains were detected
241 in Kobe I when an anti-human fibrinogen antibody was used (Figure 2, A). When an anti-
242 human fibrinogen Bβ-chain antibody was used (Figure 2, B), the single band position in
243 Kobe I was concordant with the normal Bβ-chain band in the plasma fibrinogen from a
244 healthy volunteer (normal control, NC).



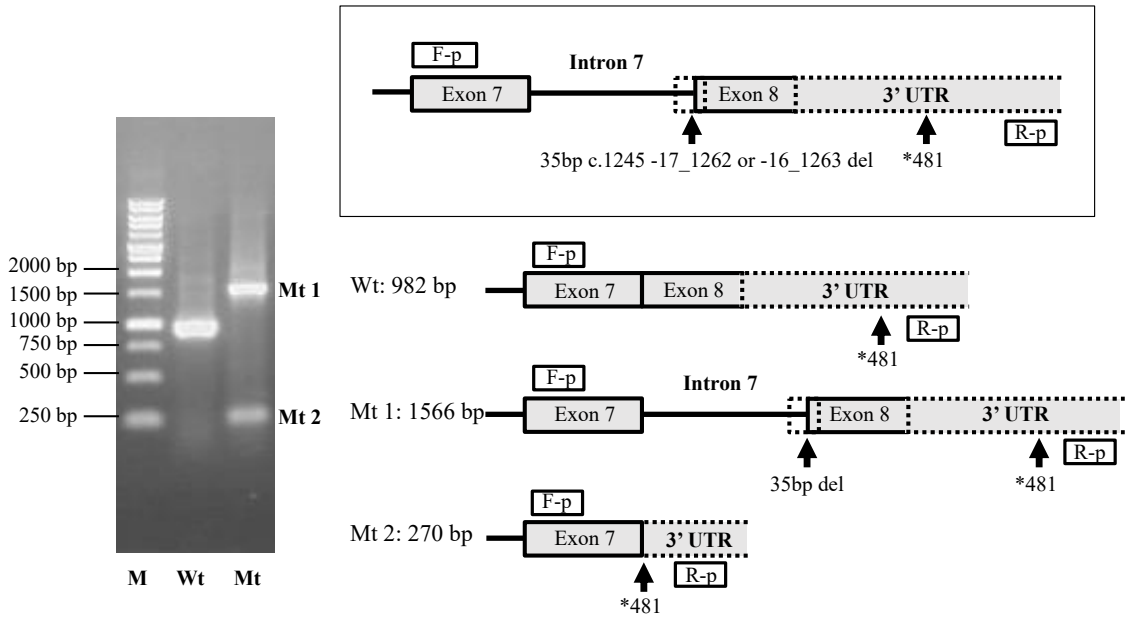
Single column

Figure 2. Western blotting analysis for plasma fibrinogen. A healthy volunteer's plasma (NC) and the patient's plasma (Pt), equivalent to 60 ng fibrinogen, were separated on 10% SDS-PAGE gels in reducing conditions. Blots were developed with an anti-fibrinogen polyclonal antibody (A) or anti-Bβ-chain polyclonal antibody (B) as described in the Materials and Methods. The bands derived from normal fibrinogen are indicated as Aα-, Bβ-, and γ-chains, and the patient band pattern corresponded to NC.

3.3. Analysis of fibrinogen Bβ-chain 35 bp deletion gene transcripts in CHO cells

In order to verify whether the nucleotide deletion at the intron 7-exon 8 boundary

influenced the transcription of mature mRNA, mutant Bβ-chain mRNA was transiently produced in CHO cells and analyzed as described in the “Materials and methods”. The construction of a minigene incorporating the 35 bp deletion is shown in Figure 3. The RT-PCR products from CHO cells transfected with a wild-type minigene showed a single band designated Wt, and those from CHO cells transfected with the mutant type minigene showed two bands, designated Mt 1 and Mt 2, respectively (Figure 3). Direct sequencing demonstrated that Wt was the normal splicing product constituting of exons 7 and 8. On the other hand, Mt 1 was an unspliced product and Mt 2 was an aberrant product spliced from intron 7 to 480 nucleotides downstream of exon 8 termination codon.



267 **Single column**

268 **Figure 3. Analysis of fibrinogen Bβ-chain mini-gene transcripts in CHO cell lines.**

269 The PCR-amplified mini-genes of the Kobe I patient were cloned into a pcDNA 3.1/V5-

270 His TOPO® vector and transfected as described in the Materials and Methods. Upper

271 right: construction of the mini-gene by amplification with forward primer (F-p) and

272 reverse primer (R-p). Left: RT-PCR products were separated on a 1% agarose gel. Lower

273 right: schematic structure predicted from sequencing for RT-PCR-amplified mini-gene

274 products. The mini-gene was constructed from exon 7 to 3'UTR. Wt: RT-PCR amplified

275 product from wild-type mini-gene-derived mRNA. Mt 1 and Mt 2: RT-PCR amplified

276 products from aberrant mini-gene-derived mRNA. M: Molecular size markers. *481: 481

277 nucleotides downstream of exon 8 termination codon.

We showed the Kobe I proposita's predicted amino acid sequence from the minigene expression results (Figure 4). The 35 bp deletion caused the splicing abnormality and resulted in the connection of 9 aberrant amino acids (Bβ416-424) or 15 aberrant amino acids (Bβ416-430) (native protein), ultimately giving a premature termination codon to both of them (Figure 4).

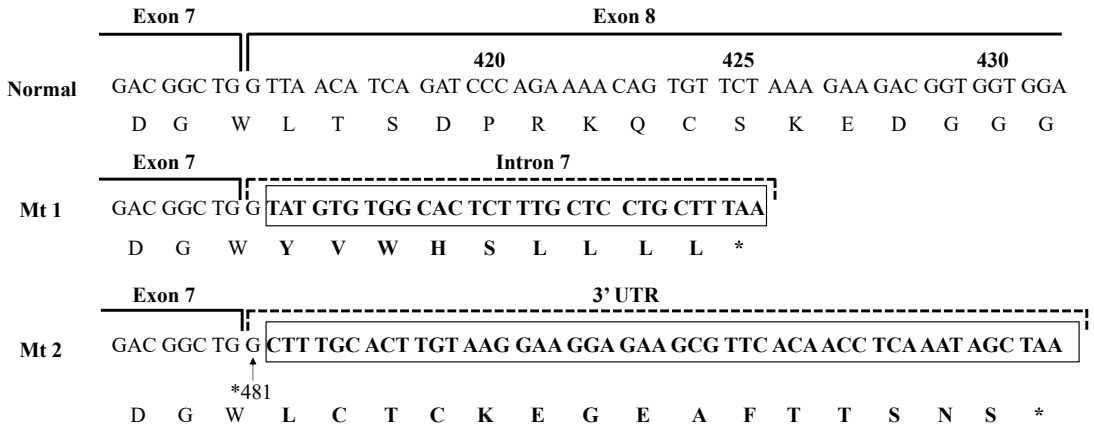


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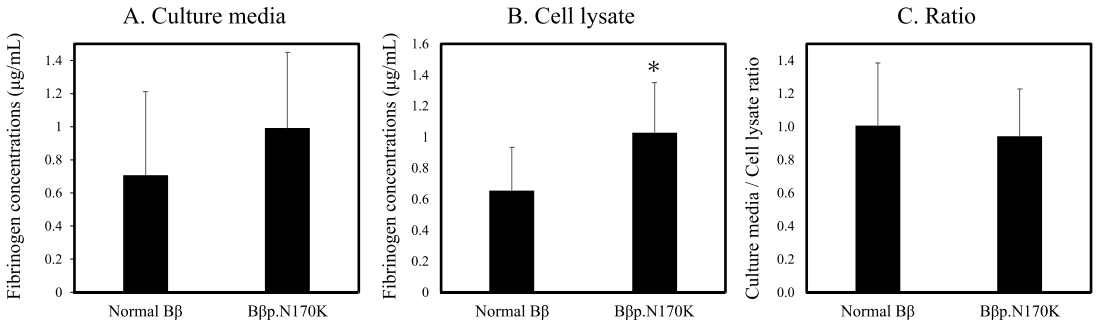
Figure 4. Predicted amino acid sequences for the mature fibrinogen protein of Kobe I. Mt 1 indicates the aberrant amino acid sequences of Bβ-combined with exon 7 and intron 7. Mt 2 indicates the aberrant amino acid sequence of Bβ-combined with exon

7 and 3'UTR (*481: 481 nucleotides downstream of exon 8 termination codon). Bold letters in the box indicate the aberrant nucleotide sequences and the bold letters without the box indicate aberrant amino acid sequences. * indicates the termination codon.

3.4. Synthesis and secretion of recombinant Bβp.N170K fibrinogen in CHO cells

We established normal Bβ and Bβp.N170K fibrinogen-expressing CHO cells. Fibrinogen concentrations in the culture media and cell lysates of fibrinogen-synthesizing cell lines were measured using ELISA. Fibrinogen concentrations as the mean ± SD and range in the culture media from normal Bβ (n = 9) and Bβp.N170K (n = 12) fibrinogen-expressing CHO cell lines were 708 ± 503 ng/mL and 990 ± 459 ng/mL, respectively (Figure 5, A). Fibrinogen concentrations in cell lysates from the Bβp.N170K fibrinogen-expressing CHO cell lines were 1029 ± 322 ng/mL, which were significantly higher than those from normal cells: 657 ± 277 ng/mL (Figure 5, B). Moreover, the fibrinogen concentration ratios of the culture media to cell lysates of the Bβp.N170K fibrinogen-expressing CHO cell lines were 0.94 ± 0.29, which were slightly lower than those from normal cells:1.00 ± 0.38 (Figure 5, C).



305 **1.5 column**

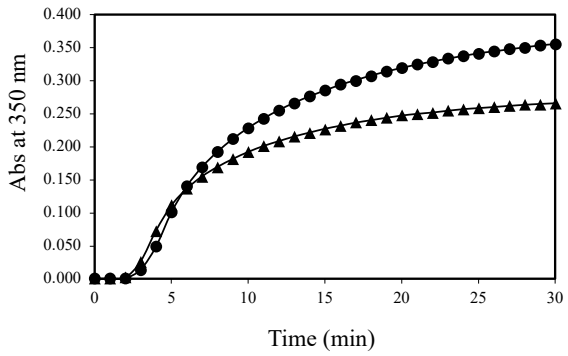
306 **Figure 5. Synthesis of Bβp.N170K fibrinogen in transfected CHO cell lines.**
307 Fibrinogen concentrations in culture media (A) and cell lysates (B) were measured using
308 ELISA as described in the Materials and Methods. The ratios of values of the culture
309 media to the cell lysate are shown in (C). Mean values are presented with standard
310 deviations indicated by error bars for BβN: n=9, Bβp.N170K: n=12. The cell lysate of
311 Bβp.N170K was significantly different from the normal control (*: $p < 0.05$).

312

313 3.5 *Thrombin-catalyzed fibrin polymerization of Kobe I plasma fibrinogen*

314 Turbidity curves of the fibrinogen purified from normal and the patient plasma were
315 obtained as shown in Figure 6, and the three calculated parameters are shown in Table 1.

316 In conditions with 0.18 mg/mL fibrinogen in the presence of 1.0 mM $CaCl_2$,
317 polymerization of Bβp.N170K fibrinogen was reduced compared with that of normal
318 plasma fibrinogen. Although the differences in lag time and Max-slope between normal
319 plasma fibrinogen and Bβp.N170K fibrinogen were not significant, the ΔAbs at 30 min
320 in Bβp.N170K fibrinogen was significantly lower than that of normal plasma fibrinogen
321 (Table 1).



322 **Single column**

323 **Figure 6. Time-dependent absorbance change at 350 nm of thrombin-catalyzed**
324 **fibrin polymerization.** The polymerization of plasma fibrinogens (0.18 mg/mL) was
325 initiated with thrombin (0.05 U/mL). We conducted experiments in triplicate, and
326 representative polymerization curves are indicated. ●: normal plasma fibrinogen, ▲:

290 Kobe I (Bβp.N170K) plasma fibrinogen.

Table 1. Three parameters of thrombin-catalyzed fibrin polymerization

	Lag time (min)		Max-slope ($\times 10^{-3}$/s)		ΔAbs (30 min)	
Normal	2.4 ± 0.3	NS	0.98 ± 0.14	NS	0.355 ± 0.010	***
Kobe I	2.2 ± 0.3		0.92 ± 0.23		0.266 ± 0.008	

Statistical analysis of each parameter between control fibrinogen and variant fibrinogens was performed Welch's T test.

NS, not significant.

***$p < .001$

4. Discussion

The proposita of Kobe I was a 31-year-old woman with continuous bleeding in the uterus after childbirth. First, we examined coagulation tests and the results showed decreased functional and antigenic fibrinogen levels with a normal functional/antigenic ratio of 0.847, suggesting hypofibrinogenemia or hypodysfibrinogenemia. Thus, we performed mutational screening and we identified two novel mutations in *FGB*: 35bp c.1245 -17_1262 or -16_1263 del and c.510T>A on different alleles.

A 35 bp deletion was located at the boundary of *FGB* intron 7-exon 8 and removed the acceptor splice site, and we expected that it may cause improper exon and intron recognition. We constructed expression vectors for the analysis of transcripts from the fibrinogen Bβ-chain gene and it resulted in the formation of two aberrant transcripts of the mutated gene. The aberrant mRNAs were unstable and degraded before translation into proteins through nonsense-mediated mRNA decay (NMD) [22] in the cytoplasm. Even if the mRNAs were not degraded through NMD, the translational products were not assembled into fibrinogen because the present study reported that residue Bβ485 is essential for fibrinogen assembly [23]. We did not detect the shortened Bβ-chain peptide in the patient's plasma by SDS-PAGE and western blotting analysis; therefore, our observation suggested that the aberrant mRNAs were destroyed through NMD before

translation. Therefore, we proved that the 35 bp deletion caused hypofibrinogenemia.

Comparing the 35 bp deletion to other cases of acceptor splice cite mutation in *FGB*, two other cases have been reported, one is *FGB* IVS4-1 G>C [24] and the other is the deletion of 4 nucleotides in *FGB* IVS6-10 to -16 [13]. The former is a point mutation of an acceptor splice site and the latter is a deletion slightly upstream of the acceptor splice site. On the other hand, the 35 bp deletion is at the boundary of intron 7-exon 8 and removes the whole acceptor splice site. Such a mutation has never been reported in fibrinogen chain genes.

There was no shortened Bβ-chain peptide that was translated from mRNA with the 35 bp deletion in the patient's plasma (Figure 2), and the assembly and secretion of the Bβp.N170K fibrinogen was almost same as normal Bβ-fibrinogen (Figure 5). Therefore, we purified Bβp.N170K fibrinogen from the patient's plasma and analyzed its function by TCFP. From this analysis, the ΔAbs (30 min) of Bβp.N170K fibrinogen was lower than that of normal plasma fibrinogen (Figure 6), it showed a delay in the lateral aggregation of protofibrils during fibrin assembly, but the extent was not so large. This was consistent with the ratio of the functional/antigenic fibrinogen value in Kobe I patient (0.847) being almost normal (0.800–1.200). Finally, we proved that Bβp.N170K caused dysfibrinogenemia.

372 The Bβ170 residue is at the middle of the coiled-coil region. According to the
373 fibrinogen variant database [web reference], some missense mutations in this part of Bβ
374 coiled-coil region, Bβp.Met148Lys, Lys178Asn, Asn190Ser, Arg196Cys, Leu202Gln,
375 Asp215Asn, and Gln189Arg (native protein), have been reported. In these missense
376 mutations, three of seven mutations, Bβp.Asn190Ser, Arg196Cys, and Asp215Asn [25-
377 27], cause dysfibrinogenemia, and the others cause hypofibrinogenemia. As for the three
378 mutations, the ratio of the functional/antigenic fibrinogen value were < 0.121, 0.684, and
379 0.440, respectively. Compared with these cases, the function of Bβp.N170K fibrinogen
380 was mildly reduced. Through amino acid substitution of Bβp.Asn190Ser and Asp215Asn,
381 N-glycosylation sites and extra oligosaccharide are introduced into the coiled-coil region
382 and these have strong negative electric charges [28]. This negative charge has a great
383 effect on the lateral aggregation of protofibrils. As for Bβp.Arg196Cys, the neo-Cys
384 residue forms a disulfide bridge with another abnormal molecule or with a Cys residue.
340 It has a substantial effect on interactions that are critical for normal lateral aggregation.
386 Thus, Bβp.N170K causes milder dysfibrinogenemia than the other three cases.
387 In conclusion, genetic analyses revealed that our patient with two novel heterozygous
388 mutations, 35 bp c.1245 -17_1262 or -16_1263 del at the boundary of *FGB* intron 7-exon
389 8 and c.510T>A in *FGB* exon 4, had hypodysfibrinogenemia, and these two mutations

were on different alleles. The experimental data indicated that aberrant splicing products derived from the former mutation caused hypofibrinogenemia, and the latter mutation caused dysfibrinogenemia due to a delay in the lateral aggregation of protofibrils during fibrin assembly. These findings suggested that the two mutations affect the fibrinogen quality and quantity in the plasma of patient Kobe I, resulting in hypodysfibrinogenemia.

Authorship

Masahiro Yoda performed all experiments and wrote the paper. Takahiro Kaido established CHO cell lines and performed the secretion experiment. Chiaki Taira, Yumiko Higuchi, Shinpei Arai and Nobuo Okumura designed the research and discussed the data. Chiaki Taira and Nobuo Okumura reviewed the paper.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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