Genetic analyses of novel compound heterozygous hypodysfibrinogenemia, Tsukuba I: *FGG* c.1129+62_65 del AATA and *FGG* c.1299+4 del A

Saki Mukai,[a,b] Kazuhiro Nagata,[a,b] Minami Ikeda,[b] Shinpei Arai,[b,c] Mitsutoshi Sugano,[b] Takayuki Honda,[c] Nobuo Okumura[a]

[a] Department of Health and Medical Sciences, 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

Address correspondence to:
Nobuo Okumura, Ph.D.
Laboratory of Clinical Chemistry
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
E-mail: nobuoku@shinshu-u.ac.jp

Word count: Abstract; 228
Manuscript; 4129 (excluding title page, Abstract, and References)
Tables: 0, Figures: 5, References: 32
Supplement Figure: 1
Supplement Figure legend: 1

Abstract

Introduction: We found a novel hypodysfibrinogenemia designated Tsukuba I caused by compound heterozygous nucleotide deletions with *FGG* c.1129+62_65 del AATA and *FGG* c.1299+4 del A on different alleles. The former was deep in intron 8 of *FGG* (IVS-8 deletion) and the latter in exon 9 of *FGG* (Ex-9 deletion), which is translated for the γ'-chain, but not the γA-chain. A Western blot analysis of plasma fibrinogen from our patient revealed an aberrant γ-chain that migrated slightly faster than the normal Bβ-chain. *Materials and Methods*: To clarify the complex genetic mechanism underlying Tsukuba I's hypodysfibrinogenemia induced by nucleotide deletions in two regions, we generated two minigenes incorporating each deletion region, transfected them into Chinese Hamster Ovary (CHO) cells, and analyzed RT-PCR products. We also established CHO cells producing the recombinant variant fibrinogen, γ'409ΔA (Ex-9 deletion). *Results and Conclusions*: Minigene I incorporating the IVS-8 deletion showed two products: a normal splicing product and the unspliced product. Minigene II incorporating the Ex-9 deletion only produced the unspliced product. The established γ'409ΔA-CHO cells secreted variant fibrinogen more effectively than normal fibrinogen. Therefore, the aberrant splicing products derived from the IVS-8 deletion cause hypofibrinogenemia most likely due to nonsense-mediated mRNA decay and the partial production of normal γA- and γ'-chains; moreover, the Ex-9 deletion causes hypodysfibrinogenemia due to the absence of normal γA- and γ'-chain production

(hypofibrinogenemia) and augmented aberrant γ'-chain production (dysfibrinogenemia).

Key words: frameshift mutation, hypodysfibrinogenemia, splicing abnormality, γA-chain, γ'-chain

Abbreviations: APTT: activated partial thromboplastin time, CHO: Chinese hamster ovary, ELISA: enzyme-linked immunosorbent assay, PAGE: polyacrylamide gel electrophoresis, PCR: polymerase chain reaction, PT: prothrombin time, RT: reverse transcriptase, SDS: sodium dodecyl sulfate

Introduction

Fibrinogen is a 340-kDa plasma glycoprotein that is composed of two sets of three different polypeptide chains: Aα, Bβ, and γ, which are linked by an extensive network of 29 intra- and inter-chain disulfide bonds and represented as $(Aα\text{-}Bβ\text{-}γ)_2$ [1]. Three chains are coded by *FGA*, *FGB*, and *FGG* (located as a cluster on chromosome 4q31), respectively [2], and synthesized, assembled into a six-chain molecule in hepatocytes, secreted into blood, and circulated at 1.8-3.5 g/l. [3]. The mature polypeptide of the Bβ-chains is composed of 461 residues, whereas two sets of *FGA* and *FGG* products exist due to alternative transcription and translation. The major Aα-chain (98-99% of the Aα-chain) contains 610 residues (translated from the first five exons of *FGA*), while the alternative Aα-chain (1-2% of the Aα-chain) contains 846 residues and is translated from all six exons of *FGA* [4]. The predominant form of the γ-chain, γA (85-93% of the γ-chain) contains 411 residues, and the variant form of the γ-chain, γ' (7-15% of the γ-chain) consists of 427 residues [5, 6]. The γA-chain is produced by the translation of all 10 *FGG* exons, while the γ'-chain is produced by alternative splicing and polyadenylation of the γ-chain mRNA transcript, resulting in the translation of exons 1-9 of *FGG* [7, 8]. A major fraction (84%) of plasma fibrinogen contains a homodimer referred to as $(Aα\text{-}Bβ\text{-}γA)_2$ or γA/γA, and the minor fraction contains a heterodimer (15% of plasma fibrinogen) referred to as (Aα-Bβ-γA) (Aα-Bβ-γ') or γA/γ' and a homodimer (1% of plasma fibrinogen) referred to as $(Aα\text{-}Bβ\text{-}γ')_2$ or γ'/γ' [5, 6].

More than 300 different mutations in the fibrinogen genes, *FGA*, *FGB*, and *FGG*, have been associated with the phenotype of afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, renal amyloidosis, or fibrinogen storage disease, as listed in the fibrinogen variant data base [9]. Hypofibrinogenemia or afibrinogenemia has been defined as reduced or negligible levels of immunologically determined fibrinogen in plasma, and dysfibrinogenemia has been defined as reduced levels of functionally determined fibrinogen with normal levels of immunologically determined fibrinogen in plasma. In the past three decades, genetic abnormalities in patients with these diseases have been detected in all three genes and identified as missense, nonsense, or frameshift mutations, splice-site abnormalities, or large deletions [9]. Hypodysfibrinogenemia, which is rarer and has the characteristics of dysfibrinogenemia and hypofibrinogenemia, has mainly been reported in *FGB* or *FGG*, and is caused by a heterozygous missense or one or two residue deletion mutations [9].

We recently identified a novel compound heterozygous variant (designated as Tsukuba I) with *FGG* c.1129+62_65 del AATA and *FGG* c.1299+4 del A, mutations on separate alleles. The former was deep in intron 8 of *FGG*, while the latter was in exon 9 of *FGG*, a region that is translated for the γ'-chain, but not the γA-chain. In order to clarify the complex genetic mechanism responsible for Tsukuba I's hypodysfibrinogenemia, we produced two minigenes incorporating each deletion region, transfected them into Chinese Hamster Ovary (CHO) cells, and analyzed RT-PCR products. We also established CHO cells producing the recombinant

variant fibrinogen, γ’409ΔA (Ex-9 deletion).

Materials and methods

This study was approved (#383) by the Ethical Review Board of Shinshu University School of Medicine. After informed consent had been obtained from the patient, blood samples were collected for biochemical and genetic analyses.

Patient and coagulation tests

The propositus of Tsukuba I was a 23-year-old man who had no history of bleeding or thrombosis. He was admitted to hospital due to the heavy use of alcohol and severe abdominal pain. Although he was a heavy user of alcohol, he was not diagnosed with alcohol abuse. Coagulation screening tests showed a low plasma fibrinogen level.

The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentrations, which were measured using the Clauss method, were evaluated 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 (Mitsubishi Chemical Medicine Co., Tokyo, Japan) [10]. The thrombin clotting time for plasma was measured without Ca ions, as described by Martinez [11].

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. In order to analyze all exons and exon-intron boundaries in the Aα-, Bβ-, and γ-chain genes, PCR and direct sequencing were performed as described elsewhere [12]. Furthermore, PCR products were subcloned into pCR2.1 plasmid vectors (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions [13].

Characterization of plasma fibrinogen

The characterization of proteins was performed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions (8% polyacrylamide gel) and reducing conditions (10% polyacrylamide gel), followed by a Western blot analysis developed with a rabbit anti-human fibrinogen antibody (DAKO, Carpinteria, CA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Medical and Biological Laboratories Ltd, Nagoya, Japan), a mouse monoclonal antibody against the human fibrinogen γ-chain (2G10, specific for γ15-35; Accurate Chemical and Scientific, Westbury, NY, USA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Medical and Biological Laboratories Ltd), or an anti-γ'-chain monoclonal antibody

(specific for γ'408-427; Upstate, Lake Placid, NY, USA) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Medical and Biological Laboratories Ltd), and enhanced with chemiluminescence detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were exposed on Hyperfilm-ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK) [14].

Construction of expression vectors for the analysis of fibrinogen γ-chain gene transcripts

DNA fragments spanning from intron 7 to exon 9 of the γ-chain gene (minigene I) and from intron 8 to the 3' untranslated region (UTR) of the γ-chain gene (minigene II) were amplified using the patient's genomic DNA and primer couples: the sense primer located in *FGG*-IVS7 (5'-CCTACGAAAGAGGGAACTTC-3') and antisense primer located in *FGG*-exon 9 (5'-TTCATGGAATACCACCGGGT-3'), or the sense primer located in *FGG*-IVS8 (5'-CTTCATAGACTTGCAGAGG-3') and antisense primer located in *FGG*-UTR (5'-GCTTTGCAAGTCCATTGTCC-3'). Purified minigene I (wild type 1; Wt1:1983 bp, mutant type 1; Mt1:1979 bp) and minigene II (Wt2:819 bp, Mt2:818 bp) were inserted into the pTARGET Vector and transfected into JM109 High Efficiency Competent Cells using the pTARGET^{TM} Mammalian Expression Vector System (Promega, Co., Madison, WI, USA) according to the manufacturer's instructions [12]. Plasmid DNAs were purified using a miniprep method and nucleotide sequences were confirmed.

Expression vectors were introduced into CHO cells using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Transfected CHO cells were cultured in 5% CO_2 at 37 °C. CHO cells were harvested 48 h after transfection. Total cellular RNA was extracted from cells using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Reverse-transcriptase (RT) reactions were performed using the oligo dT primer and RT from Moloney murine leukemia virus [13], followed by PCR amplification with two pairs of primers: the sense primer located in *FGG*-exon 8 (5'-CATGTTCAAGGTGGGACCTG-3') and antisense primer located in *FGG*-exon 9 (5'-TTCATGGAATACCACCGGGT-3') for minigene I, or the sense primer located in *FGG*-exon 9 (5'-GTGGCACTTACTCAAAAGCATC-3') and antisense primer located in *FGG*-3' UTR (5'-CTCTCTGTTCAGATAAAGTCC-3') for minigene II. RT-PCR-amplified products were separated by electrophoresis on 2% agarose gels and purified from the gels using the Gene Clean II Kit (Funakoshi, Tokyo, Japan). DNA fragments were sequenced as described above using the primers used for RT-PCR.

Expression of recombinant variant fibrinogen

Recombinant variant fibrinogen was prepared as previously described. Briefly, the variant fibrinogen γ-chain expression vector, pMLP-γ'409ΔA was altered from the pMLP-γ' plasmid (kindly provided by Lord ST, University of North Carolina), which contained

wild-type γ'-chain cDNA, by oligonucleotide-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) [14] and the following primer pair (the altered base is underlined); sense:

5'-GCCAAACAGGTC_GACCAGAGCACCCTGCG-3' and antisense:

5'-CGCAGGGTGCTCTGGTC_GACCTGTTTGGC-3' for γ'409ΔA.

The resultant mutant and normal expression vectors were co-transfected with the histidinol selection plasmid (pMSVhis) into CHO cells that expressed normal human fibrinogen Aα- and Bβ-chains (AαBβ CHO cells) using a standard calcium-phosphate co-precipitation method [15]. The cell lines were designated as γ'409ΔA- and normal (γ'N)-CHO cells, respectively. Cells were cultured and colonies were selected on histidinol (Aldrich Chem. Co. Milwaukee, WI, USA), as described elsewhere. Fibrinogen concentrations in cell lysates or culture media from the selected clones were measured by the enzyme-linked immunosorbent assay (ELISA) [10], and the assembly of fibrinogen and/or synthesis of three polypeptide chains in the cell lysate was analyzed by SDS-PAGE and a Western blot analysis as described previously [15].

Statistical analysis

The significance of differences in fibrinogen synthesis and secretion between γ' 409ΔA-, and γ'N-CHO cells was analyzed using a one-way ANOVA (analysis of variance) and

Tukey-Kramer tests. A difference was considered significant when $p < 0.05$.

Results

1. Coagulation screening tests and DNA sequence analysis

PT and APTT in the Tsukuba I patient were 12.9 (normal range: 10.0 to 13.0 seconds) and 32.8 (normal range: 23.0 to 38.0 seconds), respectively. His plasma fibrinogen concentration, measured by the Clauss method, was 0.84 g/L and that assessed by the immunological method was 1.28 g/L (normal range: 1.80 to 3.50 g/L). The thrombin clotting time for patient plasma samples (n=3) was 24.0 ± 0.5 seconds, whereas those of normal plasma samples (fibrinogen concentration of 2.92 g/L) (n=3) and fibrinogen concentration-normalized normal plasma samples (n=3) were 10.1 ± 0.2 seconds and 14.8 ± 0.2 seconds, respectively.

Sequence analysis of PCR-amplified products and subcloned PCR-amplified products revealed a compound heterozygous deletion of AATA in *FGG* intron 8; c.1129+62_65 del AATA (NCBI NM_000509.4) (called the IVS-8 deletion) and the deletion of A in *FGG* exon 9; c.1299+4 del A (called the Ex-9 deletion) (Fig. 1A and 1B). Additional PCR amplification was performed using *FGG*-exon7 and *FGG*-3'UTR primers, and the products (wild-type; 2808bp) including the IVS-8 and Ex-9 deletions were subcloned into the pCR2.1 plasmid vectors and sequenced. Sequence results revealed the presence of two clone species with IVS-8 deletion positive plus Ex-9 deletion negative (wild-type) or IVS-8 deletion negative

(wild-type) plus Ex-9 deletion positive. In brief, the mutations were on different alleles (Supplement Figure 1). Unfortunately, we did not perform the coagulation screening test or DNA sequence analysis on the parents of the patient.

2. Characterization of plasma fibrinogen.

We performed a Western blot analysis on the propositus's plasma fibrinogen. Under non-reducing conditions, an anti-human fibrinogen antibody indicated that Tsukuba I fibrinogen had a higher molecular weight than normal fibrinogen (Fig. 2A). On the other hand, under reducing conditions, four bands corresponding to the Aα-, Bβ-, γA-, and γ'-chains for normal fibrinogen, and an additional band that migrated slightly faster than the normal Bβ-chain was detected in Tsukuba I fibrinogen (Fig. 2B). As shown in Figure 2C with an anti-human fibrinogen γ-chain antibody, the additional band was clearly and abundantly present in Tsukuba I fibrinogen, indicating that the additional band was the variant γ-chain. However, an anti-human fibrinogen γ'-chain antibody (specific for γ'408-427) did not react with the variant γ-chain (Fig. 2D). The amount of the γ'-chain of Tsukuba I was smaller than that of the normal γ-chain.

3. Analysis of fibrinogen γ-chain gene transcripts in CHO cells.

In order to verify whether the nucleotide deletions of IVS-8 and Ex-9 influence the

transcription of mature mRNAs, mutant γ-chain mRNAs were transiently produced in CHO cells and analyzed as described in the “Materials and methods” section. The construction of minigene I incorporating the IVS-8 deletion was shown in Figure 3A. The RT-PCR products from CHO cells transfected with wild-type minigene I; Wt1 showed a single band that was named W1, and those from CHO cells transfected with mutant type minigene I; Mt1 showed two bands, which were named M1-1 and M1-2, respectively (Fig. 3A). Furthermore, the amount of M1-1 was larger than that of M1-2. Direct sequencing demonstrated that W1 and M1-2 were the normal splicing products constituting exons 8 and 9, whereas M1-1 was the unspliced product.

The construction of minigene II incorporating the Ex-9 deletion was shown in Figure 3B. The RT-PCR products from CHO cells transfected with wild-type minigene II; Wt2 showed a single band that was named W2, and those from CHO cells transfected with mutant type minigene II; Mt2 showed a single band, which was named M2 (Fig. 3B). Direct sequencing revealed that the mRNA of W2 was the normal splicing product of exons 9 and 10. On the other hand, the mRNA of M2 was the unspliced product.

We showed the Tsukuba I propositus’s predicted amino acid sequence from the minigene expression results (Fig. 4A and 4B). The IVS-8 deletion (c.1129+62_65 del AATA) partially caused the splicing abnormality and nucleotides in intron 9 were translated, resulting in the connection of 12 aberrant amino acids (γ351-362), ultimately giving a premature termination

codon (Fig. 4A). On the other hand, the Ex-9 deletion (c.1299+4 del A) resulted in the absence of splicing between exons 9 and 10 for the γ-chain, and only alternative translation in exon 9 was observed, leading to the production of a γ'-chain possessing 27 aberrant amino acids (γ'409-435) (Fig. 4B).

4. Synthesis and secretion of recombinant variant fibrinogen in CHO cells.

We established γ'N- and γ'409ΔA-fibrinogen-expressing CHO cells. Fibrinogen concentrations in the culture media and cell lysates of fibrinogen-synthesizing cell lines were measured by ELISA. Fibrinogen concentrations in cell lysates of the γ'409ΔA-fibrinogen-expressing cell lines (n=9) were 165 ± 66 ng/mL and significantly lower than those in the lysates of γ'normal cells: 1176 ± 534 ng/mL (n=8) (Fig. 5A). Culture media from γ'409ΔA-fibrinogen-expressing cell lines had slightly lower fibrinogen concentrations than those from normal cells (Fig. 5B); however, the fibrinogen concentration ratio of the medium/cell lysate was 5.49 ± 1.22 and significantly higher than normal (1.15 ± 0.52) (Fig. 5C).

Among the selected cell lines of variant- and normal γ'-chain-producing cells, cell lysates were analyzed by SDS-PAGE and a Western blot analysis under non-reducing and reducing conditions, as described in the "Materials and methods" section. The γ'409ΔA-fibrinogen-expressing cell line synthesized the variant γ'-chain, and the variant

γ'-chain was assembled into fibrinogen inside cells (Fig. 5D and E). Moreover, the relative molecular weight of the aberrant γ'-chain from the γ'409ΔA-fibrinogen-expressing cell line was higher than that of the normal γ'-chain.

Discussion

We identified two novel nucleotide deletions in *FGG*: compound heterozygous nucleotide deletions with c.1129+62_65 del AATA in intron 8 (IVS-8 deletion) and c.1299+4 del A in exon 9 (Ex-9 deletion), which were present on different alleles. Furthermore, a Western blot analysis of plasma fibrinogen from our patient revealed three bands of γ-chains: a normal γA-chain, aberrant γ-chain, and γ'-chain (in order of the amount of protein). Therefore, we analyzed the sequences of mRNA derived from two minigenes from our patient.

The minigene incorporating the IVS-8 deletion produced two mRNAs; one was an unspliced product, resulting in the aberrant γ-chain consisting of 362 residues (including 12 substituted residues in the C terminus). However, we did not observe the shortened length of the γ-chain for the patient's fibrinogen; therefore, this aberrant γ-chain may not exist in plasma. These results suggest that aberrant γ-chain mRNA possessing the premature termination codon at γ363 is destroyed in hepatocytes, most likely through nonsense-mediated mRNA decay [16,17]. In addition, the minigene incorporating the IVS-8 deletion partially produced a normal splicing product, but at a lower amount than that of the aberrant product.

In brief, the IVS-8 deletion may produce smaller amounts of the normal γ-chain and γ'-chain than the normal gene, resulting in hypofibrinogenemia.

Deep intronic mutations were easily detected due to advances in the development of next generation sequencing systems, resulting in an increase in reports on deficiency of coagulation factors V [18], VIII [19,20], and IX [21], and Protein S [22]. In the fibrinogen gene, only three deep intronic mutations were reported by the Sanger sequencing systems; *FGG* c.667-320A>T [23,24], *FGB* c.114+2076A>G [25], and *FGB* c.115-600A>G [26]. More than 90 % of these deep intronic mutations were one nucleotide substitutions causing the generation of a pseudo-exon (cryptic exon) in mRNA. Four-nucleotide deletions, such as Fibrinogen Tsukuba I's IVS-8 deletion (c.1129 + 62_65 del AATA), were rare among deep intronic mutations. In our search, we only found two reports of similar mutations to Tsukuba I's IVS-8 deletion: one was *CFTR* c.870-1113_-1110 del GAAT for cystic fibrosis [27] and the other *KRIT1* c.262+132_133 del AA for cerebral cavernous malformations [28], both resulting in the generation of a pseudo-exon in mRNA. However, our experiment using Tsukuba I's IVS-8 deletion minigene indicated that it did not generate a pseudo-exon, but caused incomplete splicing (partially normal splicing). These results are consistent with the findings of a computer-assisted prediction tool program (http://www.fruitfly.org/seq_tools/splice.html). Namely, when AATA was deleted in IVS-8 of *FGG*, all the splicing site prediction scores were the same as those of the wild-type, indicating

that cryptic splice sites were not generated. Therefore, our patient was a rare case of a deep intronic mutation, and the mechanism for this phenomenon needs to be clarified.

The minigene incorporating the Ex-9 deletion only produced extended exon 9 transcription corresponding to γ'-chain mRNA, whereas the splicing product combining exons 9 and 10 was absent. The aberrant γ'-chain had 435 residues (including 27 substituted and/or additional residues in the C terminus) and a larger molecular weight than the normal γ'-chain (426 residues). As a consequence, we detected a high-molecular-weight aberrant γ'-chain in the Western blot analysis of fibrinogen from the plasma of our patient. Although the mechanism underlying alternative splicing for γ'-chain mRNA production [7, 8] and the regulation of plasma γ'-chain levels have not yet been elucidated in detail, plasma fibrinogen contains approximately 7 to 15% γ'-chains and exists as three types of molecules: γA/γA (84% of total plasma fibrinogen), γA/γ' (15%), and γ'/γ' (1%) [5, 6]. The normal γ'-chain is synthesized at markedly smaller amounts than the normal γA-chain. Our results suggest that Tsukuba I patient's Ex-9 deletion may have disturbed the normal regulation of γA- and γ'-chain mRNA production, resulting in normal γA-chain mRNA being completely absent and the production of aberrant γ'-chain mRNA being strongly enhanced.

Splice-junction mutations, including splice donor sites and acceptor sites, cause a fibrinogen deficiency with splicing abnormalities, and the mutations at position +1 or +2 of donor sites are common in hypo- and/or afibrinogenemia [9]. On the other hand, the mutation at position

+4 or +5 site was rarely reported as *FGG* c.78+5G>A [29], *FGG* c.307+5G>A [30], and *FGG* c.307+4A>G [31].

Furthermore, we established γ’409ΔA-fibrinogen-expressing CHO cells, and ELISA and Western blot analyses of cell lysates and conditioned media indicated that the aberrant γ’-chain was synthesized and assembled into fibrinogen, and then secreted more efficiently than normal control fibrinogen. These results are also consistent with the presence of the aberrant γ-chain in the plasma of our Tsukuba I patient, and at an amount that was slightly smaller than that of the normal γ-chain and markedly larger than that of the normal γ'-chain. In brief, Tsukuba I patient’s Ex-9 deletion may lead to hypofibrinogenemia.

Functional and immunological assessments of fibrinogen concentrations and thrombin clotting times revealed that the Fibrinogen Tsukuba I patient had hypodysfibrinogenemia. Huang et al. reported four heterozygous dysfibrinogenemia patients with *FGG* c.1299+1 del G in a Chinese pedigree [32]. The proposita’s plasma fibrinogen concentration, measured by the Clauss method, was 0.9 g/L, while that evaluated by the immunological method was 2.8 g/L. The *FGG* c.1299+1 del G variant may have a 408 Ser followed by DQSTLRKQNMTHFTLRMICRKLTANFY. The γ'-chain of the *FGG* c.1299+1 del G variant co-migrated with the Bβ-chain and the patient’s plasma fibrinogen indicated severely declined thrombin-induced fibrin polymerization. The overall negative charge of the 20 γ'-chain C-terminal residues was substituted by 28 positively charged aberrant γ'-chain C-terminal

residues (Fig. 4B). This substitution may have led to Tsukuba I patient's impaired thrombin clotting time and lower fibrinogen concentration by the Clauss method.

In conclusion, genetic analyses revealed that our compound heterozygous patient with c.1129+62_65 del AATA in *FGG* intron 8 and c.1299+4 del A in *FGG* exon 9 (Tsukuba I) had hypodysfibrinogenemia. Aberrant splicing products derived from the former mutation caused hypofibrinogenemia, most likely due to nonsense-mediated mRNA decay and the partial production of the normal γA- and γ'-chain. In addition, the latter mutation caused hypofibrinogenemia due to the absence of normal γA- and γ'-chain production and also caused dysfibrinogenemia due to augmented aberrant γ'-chain production.

Authorship

Saki Mukai and Kazuhiro Nagata performed the research, analyzed the data, and wrote the manuscript. Minami Ikeda analyzed DNA sequences. Shinpei Arai, Mitsutoshi Sugano, Takayuki Honda, and Nobuo Okumura designed the research and discussed the data. N Okumura reviewed the manuscript.

Conflict of Interest Statement

The authors state that they have no conflicts of interest.

Acknowledgments

We gratefully acknowledge Dr. Yuichi Hasegawa (Tsukuba University Graduate School of Medicine) for the patient referral of Tsukuba I.

References

[1] J.W. Weisel, Fibrinogen and fibrin, Adv. Protein Chem. 70 (2005) 247–299.

[2] J.A. Kant, A.J. Fornace Jr., D. Saxe, M.I. Simon, O.W. McBride, G.R. Crabtree, Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 2344–2348.

[3] J.W. Weisel, R.I. Litvinov, Mechanisms of fibrin polymerization and clinical implications, Blood 121 (2013) 1712–1719.

[4] Y. Fu, G. Grieninqer, Fib420: a normal human variant of fibrinogen with two extended alpha chains, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 2625-8.

[5] D.A. Meh, K.R. Siebenlist, M.W. Mosesson, Identification and characterization of the thrombin binding sites on fibrin, J. Biol. Chem. 271 (1996) 23121-5.

[6] C. Wolfenstein-Todel, M.W. Mosesson, Human plasma fibrinogen heterogeneity: evidence for an extended carboxyl-terminal sequence in a normal gamma chain variant (gamma'), Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 5069-73.

[7] D.W. Chung, E.W. Davie, Gamma and gamma' chains of human fibrinogen are produced by alternative mRNA processing, Biochemistry. 23 (1984) 4232-6.

[8] A.J. Fornace. Jr, D.E. Cummings, C.M. Comeau, J.A. Kant, G.R. Crabtree, Structure of the human gamma-fibrinogen gene. Alternate mRNA splicing near the 3' end of the gene produces gamma A and gamma B forms of gamma-fibrinogen, J. Biol. Chem. 259 (1984) 12826-30.

[9] Groupe d'Etude sur 1'Hémostase et la Thrombose, Base de données des variants du FibrinogèneGEHT Web site. http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html, (accessed 15/7/2016).

[10] N. Okumura, K. Furihata, F. Terasawa, R. Nakagoshi, I. Ueno, T. Katsuyama, Fibrinogen Matsumoto I: a gamma 364Asp → His (GAT→CAT) substitution associated with

defective fibrin polymerization, Thromb. Haemost. 75 (1996) 887-91.

[11] J. Martinez, RR. Holburn, SS. Shapiro, AJ. Erslev, Fibrinogen Philadelphia. A hereditary hypodysfibrinogenemia characterized by fibrinogen hypercatabolism, J Clin Invest. 53 (1974) 600-11.

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

[13] F. Terasawa, Y. Kamijyo, N. Fujihara, K. Yamauchi, T. Kumagai, T. Honda, S. Shigematsu, N. Okumura, In vitro transcription of compound heterozygous hypofibrinogenemia Matsumoto IX; first identification of FGB IVS6 deletion of 4 nucleotides and FGG IVS3-2A>G causing abnormal RNA splicing, Clin. Chim. Acta. 411 (2010) 1325-9.

[14] N. Fujihara, A. Haneishi, K. Yamauchi, F. Terasawa, T. Ito, F. Ishida, N. Okumura, A C-terminal amino acid substitution in the gamma-chain caused by a novel heterozygous frameshift mutation (Fibrinogen Matsumoto VII) results in hypofibrinogenaemia, Thromb. Haemost. 104 (2010) 213-223.

[15] N. Okumura, F. Terasawa, H. Tanaka, M. Hirota, H. Ota, K. Kitano, et al, Analysis of fibrinogen gamma-chain truncations shows the C-terminus, particularly γIle387, is essential for assembly and secretion of this multichain protein, Blood. 99 (2002) 3654-60.

[16] K. Soya, Y. Takezawa, N. Okumura, F. Terasawa, Nonsense-mediated mRNA decay was demonstrated in two hypofibrinogenemias caused by heterozygous nonsense mutations of FGG, Shizuoka III and Kanazawa II, Thromb. Res. 132 (2013) 465-70.

[17] J.N. Miller, D.A. Pearce, Nonsense-mediated decay in genetic disease: friend or foe?, Mutat. Res. Rev. Mutat. Res. 762 (2014) 52-64.

[18] E. Castoldi, C. Duckers, C. Radu, L. Spiezia, V. Rossetto, G. Tagariello, et al, Homozygous F5 deep-intronic splicing mutation resulting in severe factor V deficiency and undetectable thrombin generation in platelet-rich plasma, J. Thromb. Haemost. 9 (2011) 959-68.

[19] C. Castaman, S.H. Giacomelli, M.E. Mancuso, G. D'Andrea, R. Santacroce, S. Sanna, et al, Deep intronic variations may cause mild hemophilia A, J. Thromb. Haemost. 9 (2011) 1541-8.

[20] B. Pezeshkpoor, N. Zimmer, N. Marquardt, I. Nanda, T. Haaf, U. Budde, et al, Deep intronic 'mutations' cause hemophilia A: application of next generation sequencing in patients without detectable mutation in F8 cDNA, J. Thromb. Haemost. 11 (2013) 1679-87.

[21] J. Feng, Q. Liu, J. Drost, S.S. Sommer, Deep intronic mutations are rarely a cause of hemophilia B, Hum. Mutat. 14 (1999) 267-8.

[22] G. D'Andrea, P. Di. Perna, V. Brancaccio, E.M. Faioni, G. Castaman, G. Cibelli, et al, A novel G-to-A mutation in intron-N of the protein S gene leading to abnormal RNA splicing in a patient with protein S deficiency, Haematologica. 88 (2003) 459-64.

[23] M. Platè, S. Duga, G. Castaman, F. Rodeghiero, R. Asselta, Recurrence of the 'deep-intronic' FGG IVS6-320A>T mutation causing quantitative fibrinogen deficiency in the Italian population of Veneto, Blood. Coagul. Fibrinolysis. 20 (2009) 381-4.

[24] S. Spena, R. Asselta, M. Platé, G. Castaman, S. Duga, M.L. Tenchini, Pseudo-exon activation caused by a deep-intronic mutation in the fibrinogen gamma-chain gene as a novel mechanism for congenital afibrinogenemia, Br. J. Haematol. 139 (2007) 128-32.

[25] A. Dear, J. Daly, S.O. Brennan, A. Tuckfield, P.M. George, An intronic mutation within FGB (IVS1+2076 a>g) is associated with afibrinogenemia and recurrent transient ischemic attacks, J. Thromb. Haemost. 4 (2006) 471-2.

[26] R.L. Davis, V.M. Homer, P.M. George, S.O. Brennan, A deep intronic mutation in *FGB*

creates a consensus exonic splicing enhancer motif that results in afibrinogenemia caused by aberrant mRNA splicing, which can be corrected in vitro with antisense oligonucleotide treatment, Hum. Mutat. 30 (2009) 221-7.

[27] C. Costa, V. Pruliere-Escabasse, A. de Becdelievre, C. Gameiro, L. Golmard, C. Guittard, et al, A recurrent deep-intronic splicing CF mutation emphasizes the importance of mRNA studies in clinical practice, J. Cyst. Fibros. 10 (2011) 479-82.

[28] F. Riant, S. Odent, M. Cecillon, L. Pasquier, C. de Baracé, M.P. Carney, et al, Deep intronic KRIT1 mutation in a family with clinically silent multiple cerebral cavernous malformations, Clin. Genet. 86 (2014) 585-8.

[29] R. Asselta, S. Duga, T. Simonic, M. Malcovati, E. Santagostino, P.L.F. Giangrande, et al, Afibrinogenemia: first identification of splicing mutation in the fibrinogen gamma chain gene leading to a major gamma chain truncation, Blood. 96 (2000) 2496-500.

[30] M. Margaglione, R. Santacroce, D. Colaizzo, D. Seripa, G. Vecchione, M.R. Lupone, et al, A G-to-A mutation in IVS-3 of the human gamma fibrinogen gene causing afibrinogenemia due to abnormal RNA splicing, Blood. 96 (2000) 2501-5.

[31] R. Asselta, M. Platè, M. Robusto, M. Borhany, I. Guella, G. Soldà, et al, Clinical and molecular characterisation of 21 patients affected by quantitative fibrinogen deficiency, Thromb. Haemost. 113 (2015) 567-76.

[32] D. Huang, H. Chen, X. Hu, X. Wang, H. Wang, Identification of a novel splicing mutation in the fibrinogen gamma chain gene leading to dysfibrinogenaemia in a Chinese pedigree, Pathology. 47 (2015) 145-50.

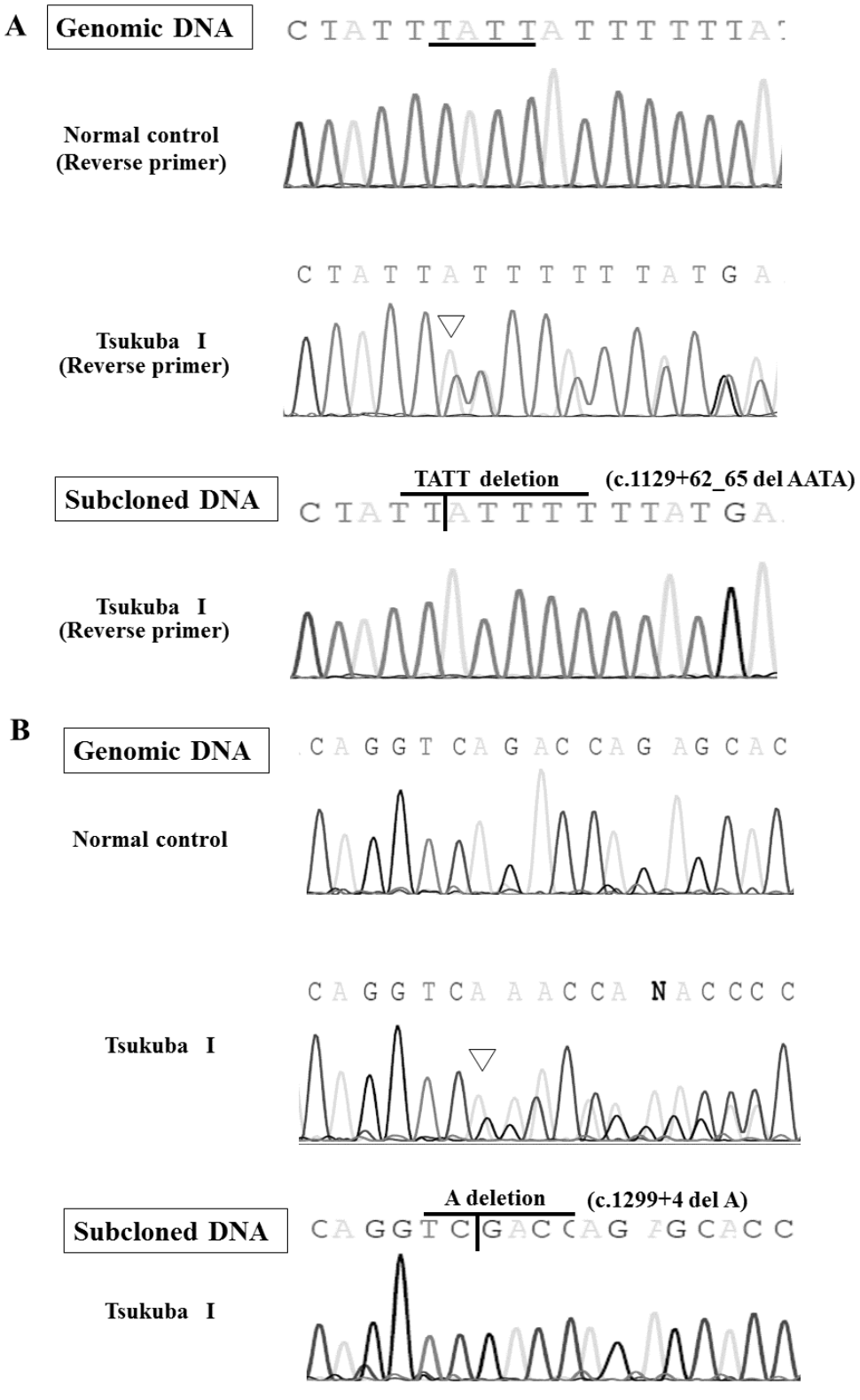

A
Genomic DNA
Normal control
(Reverse primer)
Tsukuba I
(Reverse primer)
TATT deletion
(c.1129+62_65 del AATA)
Subcloned DNA
Tsukuba I
(Reverse primer)
B
Genomic DNA
Normal control
Tsukuba I
A deletion
(c.1299+4 del A)
Subcloned DNA
Tsukuba I

Figure 1. Nucleotide sequences of fibrinogen γ-chain intron 8 (A) and exon 9 (B) in Tsukuba I.

The PCR-amplified γ-chain genes in the Tsukuba I propositus were sequenced (Genomic DNA). Wild-type and mutant-type genes were cloned and sequenced (Subcloned DNA). Sequence reactions were performed using the reverse primer (A) or forward primer (B). Sequence results indicate *FGG* c.1129+62_65 del AATA (sense sequence) (A) and c.1299+4 del A (B), respectively.

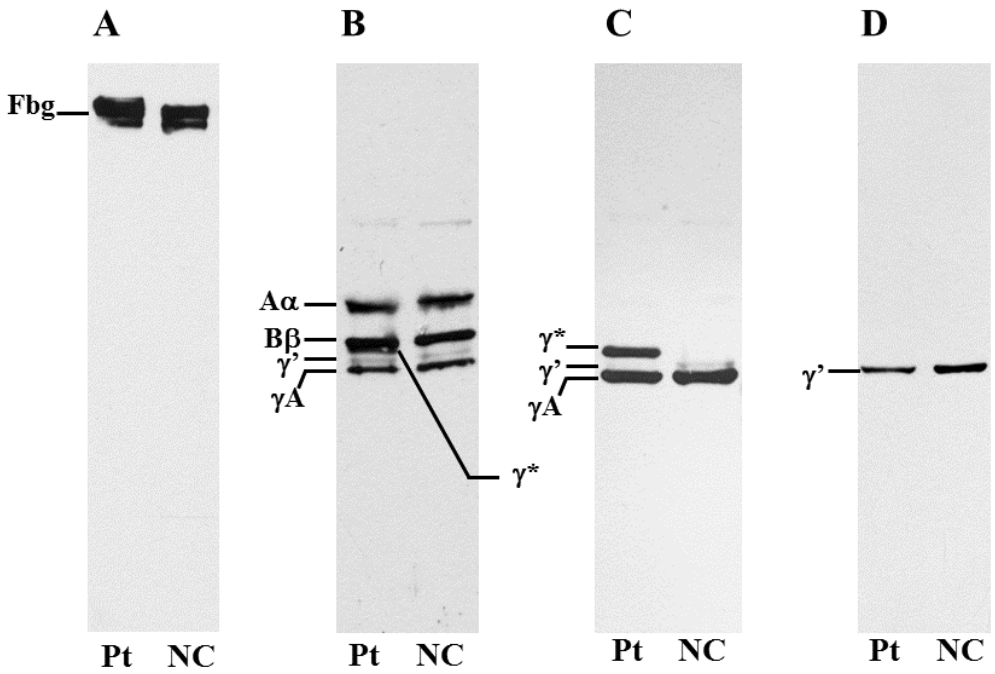


Figure 2. Western blot analysis of plasma fibrinogen.

A healthy volunteer's plasma (NC) and the patient's plasma (Pt), equivalent to 15 ng (A), 40 ng (B, C), and 240 ng (D) fibrinogen, were separated on 8% SDS-PAGE under non-reducing conditions (A) or 10% SDS-PAGE under reducing conditions (B-D). Blots were developed with an anti-fibrinogen polyclonal antibody (A, B), anti-γ-chain monoclonal antibody (2G10) (C), or anti-γ'-chain monoclonal antibody (D) as described in the "Materials and Methods" section. The bands derived from normal fibrinogen are indicated as Aα, Bβ, γA, and γ', and the extra band derived from the patient's fibrinogen was γ*.

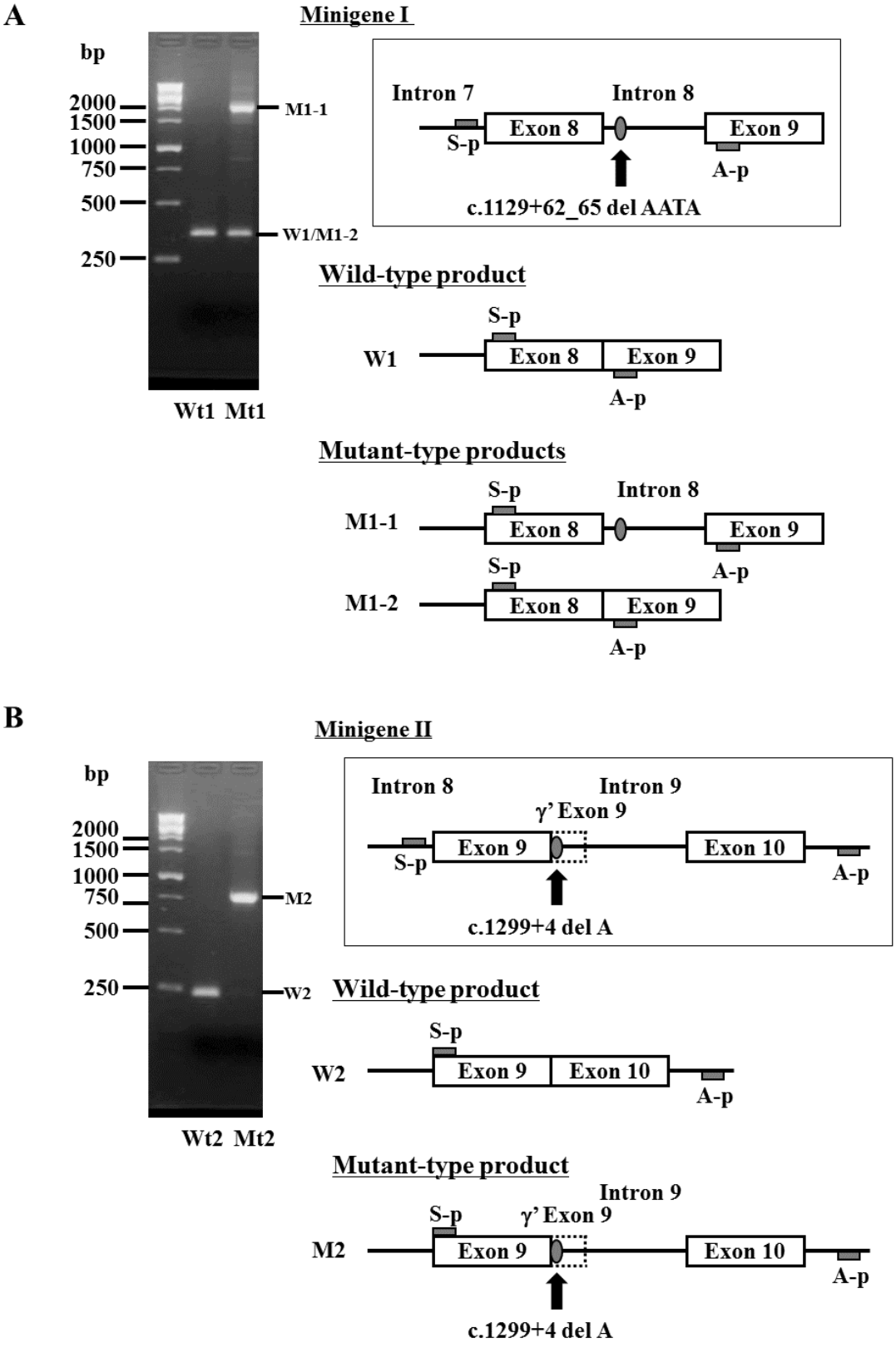

A
Minigene I
bp
2000
1500
1000
750
500
250
M1-1
W1/M1-2
Wt1 Mt1
Intron 7
Intron 8
Exon 8
Exon 9
S-p
A-p
c.1129+62_65 del AATA
Wild-type product
S-p
W1
Exon 8
Exon 9
A-p
Mutant-type products
S-p
Intron 8
M1-1
Exon 8
Exon 9
A-p
S-p
M1-2
Exon 8
Exon 9
A-p
B
Minigene II
bp
2000
1500
1000
750
500
250
M2
W2
Wt2 Mt2
Intron 8
Intron 9
γ' Exon 9
Exon 9
Exon 10
S-p
A-p
c.1299+4 del A
Wild-type product
S-p
W2
Exon 9
Exon 10
A-p
Mutant-type product
Intron 9
S-p
γ' Exon 9
M2
Exon 9
Exon 10
A-p
c.1299+4 del A

Figure 3. Analysis of fibrinogen γ-chain minigene transcripts in CHO cells.

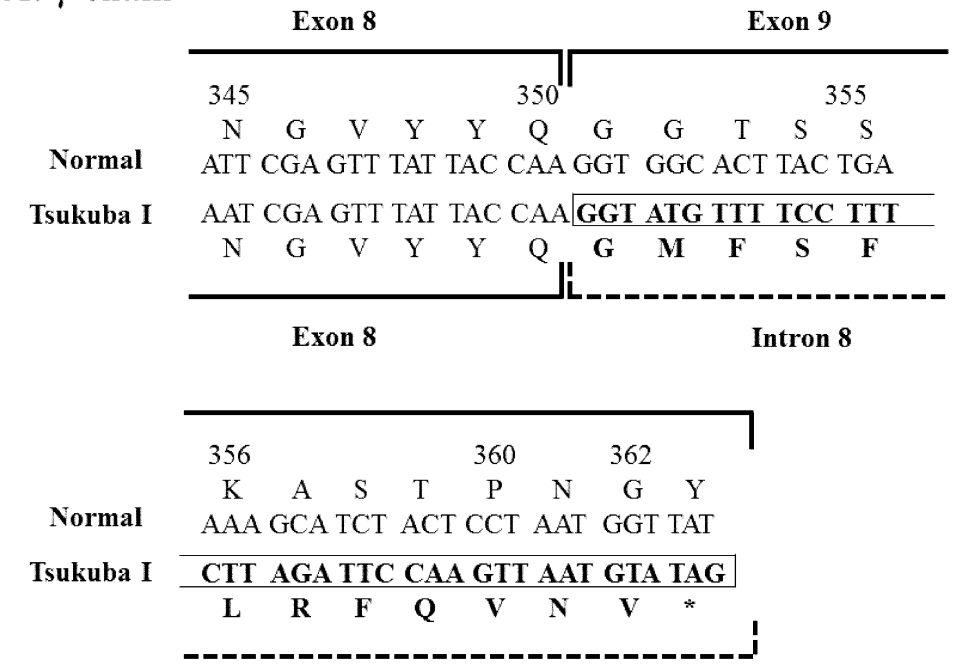
The PCR-amplified minigenes of Tsukuba I propositus were cloned into the pTARGET vector and transfected into CHO cells. RT-PCR amplified minigene products were sequenced, as described in the "Materials and Methods" section.

(A), (B) *upper right*: Construction of the minigene by amplification with the sense primer (S-p) and antisense primer (A-p). (A), (B) *left*: RT-PCR products were separated on a 2% agarose gel. (A), (B) *lower right*: Schematic structure predicted from sequencing for RT-PCR-amplified minigene products.

(A): Minigene I was constructed from *FGG* intron 7 to exon 9. Lane M: DNA size marker. Wt1: RT-PCR-amplified products from wild-type minigene I-derived mRNA. Mt1: RT-PCR-amplified products from aberrant minigene I-derived mRNA. W1 was mRNA from wild-type minigene I. M1-1 was mRNA from aberrant minigene I, which is the unspliced product. M1-2 was the normal splicing product derived from aberrant minigene 1.

(B): Minigene II was constructed from *FGG* intron 8 to the *FGG* 3'-flanking region. Wt2: RT-PCR-amplified products from wild-type minigene II-derived mRNA. Mt2: RT-PCR-amplified products from aberrant minigene II-derived mRNA. W2 was mRNA from wild-type minigene II. M2 was mRNA from aberrant minigene II, which is the unspliced product.

A: γ-chain



B: γ'-chain

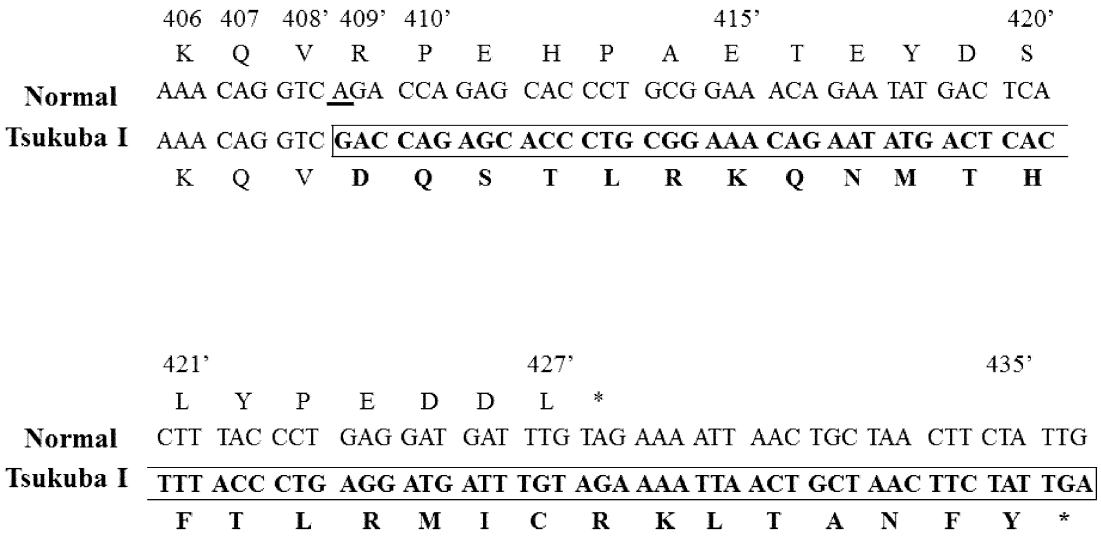


Figure 4. Tsukuba I propositus's predicted amino acid sequences.

(A) indicates the aberrant amino acid sequence of the γ-chain combined with exon 8 and intron 9. (B) indicates the aberrant amino acid sequence of the γ'-chain with c.1299+4 del A. The bold letters in the box indicate the aberrant nucleotide sequence and the bold letters without the box indicate the aberrant amino acids sequence. * indicates the terminal codon.

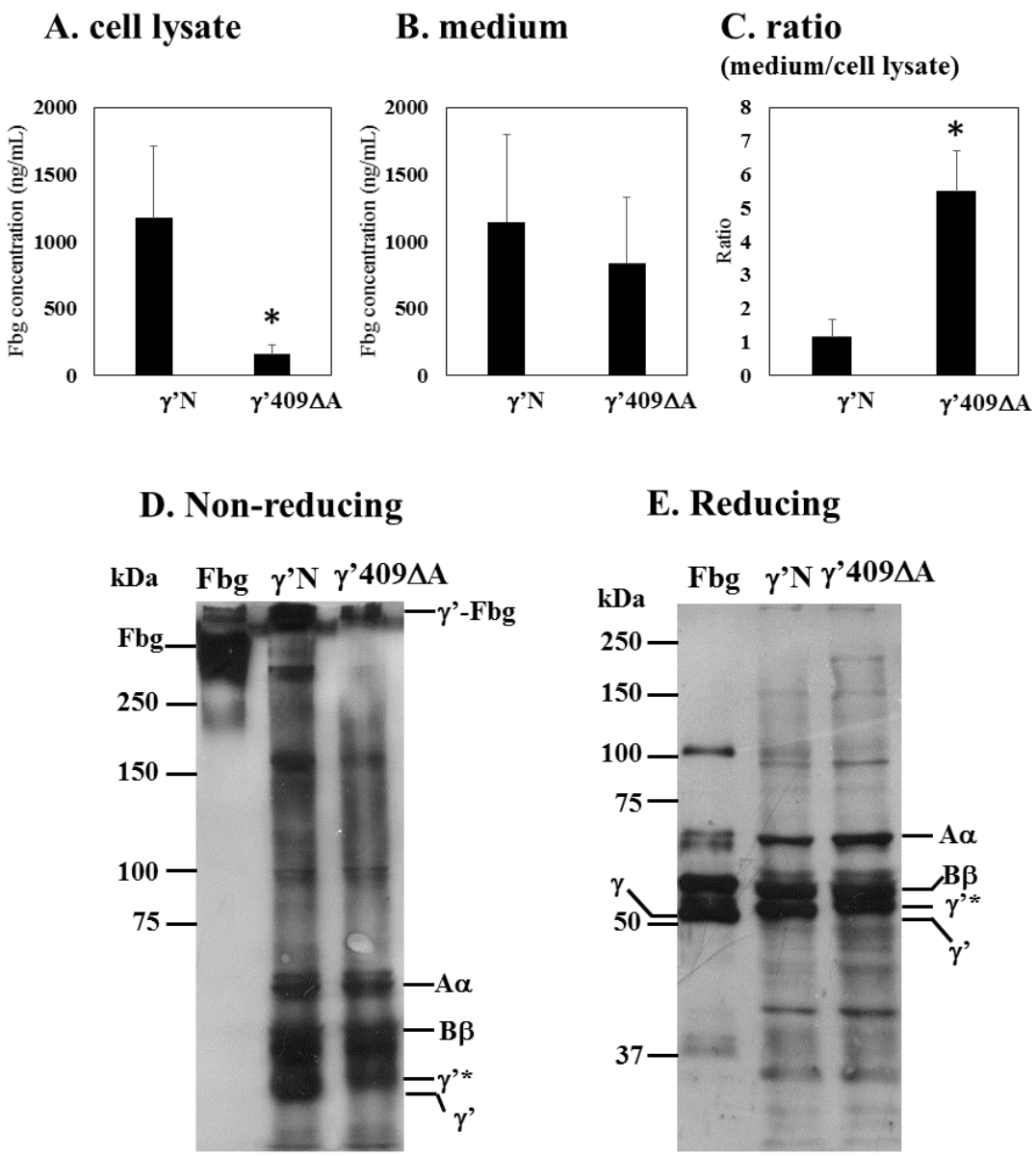
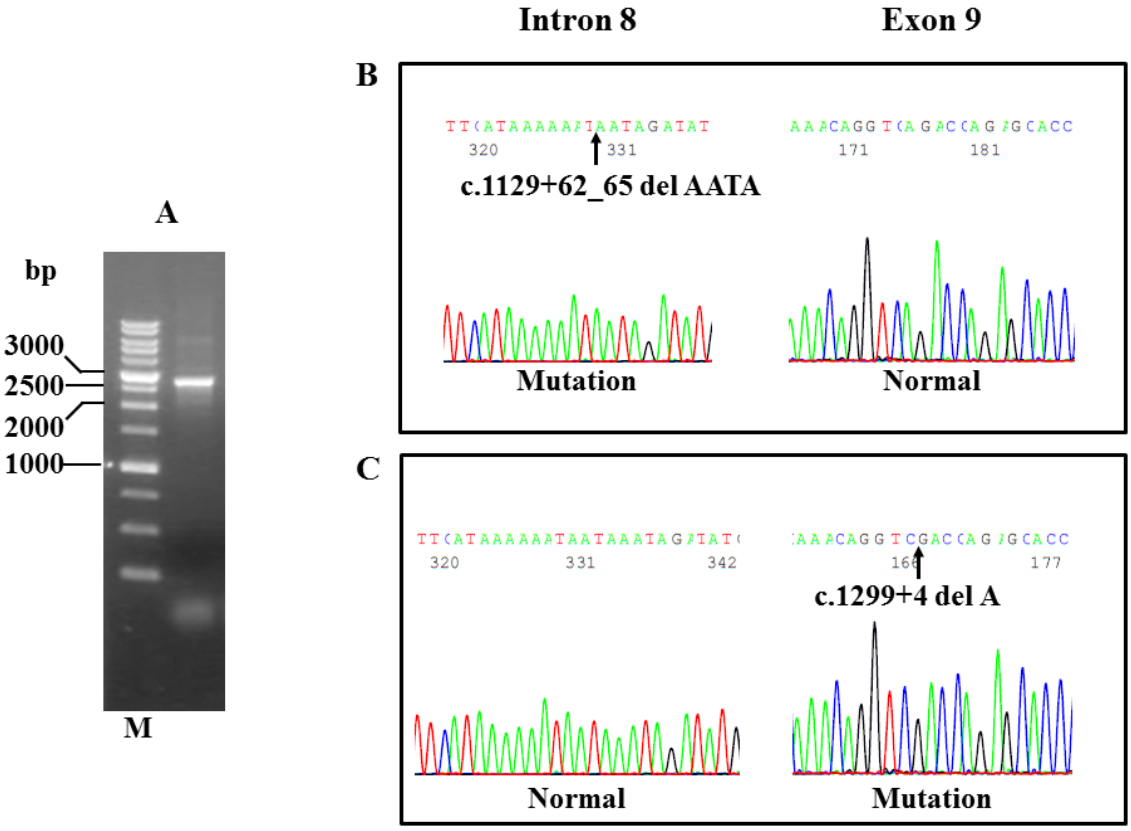

A. cell lysate
B. medium
C. ratio
(medium/cell lysate)
Fbg concentration (ng/mL)
2000
1500
1000
500
0
*
γ'N
γ'409ΔA
Ratio
8
7
6
5
4
3
2
1
D. Non-reducing
E. Reducing
kDa
Fbg
γ'N
γ'409ΔA
γ'-Fbg
250
150
100
75
Aα
Bβ
γ'*
γ'
γ
50
37

Figure 5. Synthesis of variant fibrinogen in transfected CHO cells.

Fibrinogen concentrations in cell lysates (A) and culture media (B) were measured by ELISA, as described in the “Materials and Methods” section. The ratios of the values of the culture medium to the cell lysate are shown in panel C. Mean values are presented with standard deviations indicated by error bars for γ’normal (γ’N): n=8, γ’409ΔA: n=9. The cell lysate and ratio of the variant were significantly different from the normal (*: $p < 0.01$).

(D), (E): A Western blot analysis of cell lysates. Samples from cell lysates were subjected to 8% SDS-PAGE under non-reducing conditions (D) or 10% SDS-PAGE under reducing conditions (E). The blots were reacted with an anti-fibrinogen antibody and detected by chemiluminescence, as described in the “Materials and methods” section. Lane Fbg: purified fibrinogen from normal plasma, lane γ'N: γ’N-CHO cells, and lane γ’409ΔA: γ’409ΔA-CHO cells. The aberrant γ'-chain synthesized in γ’409ΔA-CHO cells is indicated as γ'*. The left sides of panels D and E show the molecular size markers.



Supplement Figure 1. Nucleotide sequences of subcloning DNA amplified the regions including the IVS-8 and Ex-9 deletions.

PCR products amplified with sense primer located in *FGG*-IVS7 and antisense primer located in *FGG*-UTR, and separated on a 1% agarose gel (A). Lane M: DNA size marker. PCR products (2808 bp) were subcloned into pCR2.1 plasmid vectors, according to the manufacturer's instructions. Sequence analyses were performed for several clones and obtained two species. One was c.1129+62_65 del AATA mutation and normal sequence of exon 9 (B), and another was normal sequence of intron 8 and c.1299+4 del A mutation (C). These sequence results revealed that the two mutations were on different alleles.