Molecular analysis of afibrinogenemic mutations caused by a homozygous FGA1238 bp deletion, and a compound heterozygous FGA1238 bp deletion and novel FGA c.54+3A>C substitution

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SUMMARY

Introduction: We identified two afibrinogenemic girls in two Japanese families and performed molecular analysis to clarify the mechanisms of fibrinogen defects.

Methods: Genetic analyses were performed by PCR amplification of the fibrinogen gene and DNA sequence analysis. To analyze the mechanisms of mature fibrinogen defects in plasma, we cloned minigenes from the proposita's PCR-amplified DNA, transfected them into CHO cells, and sequenced the cDNA amplified with the RT reaction followed by PCR.

Results: Sequence analyses indicated that one was caused by a homozygous 1238 bp deletion of the fibrinogen Aα-chain gene (FGAΔ1238) and the other was a compound heterozygous FGAΔ1238 and novel FGA c.54+3A>C substitution. The minigene corresponding to FGAΔ1238 generates two aberrant mRNAs, both of which may induce a frameshift and terminate prematurely. In contrast, the minigene corresponding to FGA c.54+3A>C generates two aberrant mRNAs, one of which may induce a frameshift and terminate prematurely, and the other uses a cryptic 5' splice site in exon 1, resulting in the deletion of six amino acids in signal peptides.

Conclusion: Molecular analyses of both genetic variants suggest that the lack of a mature Aα-chain, impaired assembly, and/or secretion of the fibrinogen molecule may lead to afibrinogenemia.

Keywords: afibrinogenemia, Aα-chain, splicing abnormality, deletion, compound heterozygote

INTRODUCTION

Fibrinogen is a 340 kDa plasma glycoprotein composed of two sets of three different polypeptide chains (Aα, Bβ, and γ), and it is expressed as $(Aα, Bβ, γ)_2$. Each chain is synthesized, assembled into a six-chain molecule in hepatocytes, secreted into the blood, and circulated at 1.8-3.5 g/l [1,2]. Aα-, Bβ- and γ- chains in plasma fibrinogen are composed of 610, 461 and 411 amino acid residues, which are composed of 3 genes coding for fibrinogen beta (*FGB*), fibrinogen alpha (*FGA*), and fibrinogen gamma (*FGG*), clustered in a region of approximately 50 kb on chromosome 4q31.3 [3]. Genetic mutations in fibrinogen chain genes have been associated with afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia, as listed in the fibrinogen variant database [updated on 26/01/2012, http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Base-de-donnees/Base-de-donnees-des-variants-du-Fibrinogene_40_.html.]

Afibrinogenemia is a life-long bleeding disorder associated with the complete deficiency of plasma fibrinogen; however, the prevalence of afibrinogenemia is lower than 1 in 1 million. The severity of the bleeding tendency varies in afibrinogenemic patients. The most typical symptom is umbilical cord bleeding and other relatively frequent symptoms are hemorrhage from the mucosal tracts, hemarthroses, and hematomas, although life-threatening bleeding such as splenic rupture, gastrointestinal hemorrhage and central nervous system bleeding may be rare [4,5].

Genetic abnormalities and the molecular bases in patients with afibrinogenemia have been found in all three genes and identified as missense, nonsense, or frameshift mutations; splice-site abnormalities; or large deletions [4-8]. In particular, reports

causing large deletions between 1.2 and 15 kb were restricted in *FGA* (fibrinogen variant database). Recently, we identified two afibrinogenemias, caused by homozygous deletion of 1238 bases of the Aα-chain gene (*FGA*Δ1238) and compound heterozygous deletion of *FGA*Δ1238 and *FGA* c.54+3A>C, examined the molecular basis of the lack of protein, and designated them as Fibrinogen Yokkaichi and Fibrinogen Kurashiki II, respectively.

MATERIALS AND METHODS

Patients and sampling of plasma and DNA

The Yokkaichi proposita was a 4-month-old girl who was found to have persistent omphalorrhagia 6 days after birth and progressed to intradural hematoma and hemorrhagic shock. Her fibrinogen level was less than the detection limit and she was diagnosed with afibrinogenemia and was drip infused with fibrinogen concentrates once every three days until the intradural hematoma disappeared. Now, fibrinogen concentrate is used only when she has a bleeding event. The parents had no obvious consanguinity and none of her family had a bleeding tendency or thrombotic complications.

The Kurashiki-II proposita was a 1-year-old girl, who was hospitalized with infectious enterogastritis, and a hematoma developed at the antibiotic drip infusion site; her blood coagulation screening test indicated impaired clot formation. She was drip infused with fibrinogen concentrates during hospitalization. The parents had no consanguinity and none of her family had a bleeding tendency or thrombotic complications.

Blood was collected from both propositae and parents with informed consent for coagulation and genetic analyses. Nine volumes of blood were collected into plastic tubes containing 1 volume of 3.2% trisodium citrate. Separated plasma was used for coagulation tests and buffy coat cells were extracted to prepare genomic DNA [9]. This analysis was approved by the Ethics Committee for Genetic Analysis of Shinshu University School of Medicine.

Coagulation screening tests

Blood coagulation screening tests; prothrombin time (PT), activated partial thromboplastine time (APTT), the fibrinogen concentration, which was determined by the thrombin time method, and the immunologic fibrinogen concentration were measured as described elsewhere [9].

DNA sequence analysis and long-range polymerase chain reaction (PCR)

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 [9]. To examine the large deletion of the genes, long-range PCR for the entire Aα-, Bβ-, and γ-chain genes was performed using the TaKaRa LA Taq (Takara Bio Inc., Otsu, Japan) and the pair of primers for Aα-chain: sense primer: 5'-CAGCTAGCTTACCTAAGCACC-3' and antisense primer: 5'-GTTAAGGAAGAAATGCAAGGG-3', Bβ-chain: sense primer: 5'-GGATGGTTTCTTGGAGC-3' and antisense primer: 5'-ACGTCTGCTTGAGAGTTTTAG-3', and γ-chain: sense primer: 5'-GAACTGGGACATGGGGGAAGT-3' and antisense primer:

5’-GCTTTGCAAGTCCATTGTC-3’. Amplification was performed with 1 μg genomic DNA and PCR conditions were as follows: an initial denaturation step at 94 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 sec and annealing at 66 °C for 15 min, and final extension at 72 °C for 10 min. PCR products were electrophoresed on a 1.0 % agarose gel and stained with ethidium bromide.

Construction of expression vectors

To amplify the DNA fragments spanning from intron 2 to exon 5 including *FGA*Δ1238 and from 5’ untranslated region (UTR) to intron 2 including intron 1 point mutation of the Aα-chain gene (*FGA* c.54+3A>C), PCR was performed using the Kurashiki II patient’s genomic DNA and the primers: sense primer located in *FGA*-IVS2 (5’-GAAAGGGTAGGAAGAAATGGGG-3’) and antisense primer located in *FGA*-exon 5 (5’-CCTGGGGCTTTCCGTCTCTGA-3’), or sense primer located in *FGA*-UTR (5’-CAGCTAGCTTACCTAAGCACC-3’) and antisense primer located in *FGA*-IVS2 (5’-CACCCACTAATGATCCAGAGTTGG-3’). Purified DNA fragments including *FGA*Δ1238 (Mt1: 1258 bp), *FGA* c.54+3A (Wt2: 1826 bp), and *FGA* c.54+3C (Mt2: 1826 bp) were inserted into pTARGET Vector (Promega, Co., Madison, WI, USA) and transfected into JM109 High Efficiency Competent Cells (Promega) according to the manufacturer’s instructions. Plasmid containing wild- or mutant-type DNA fragment (named as pT-Aα-Mt1, pT-Aα-Wt2, or pT- Aα-Mt2 vector, respectively) was purified using a Qiagen Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany) and the nucleotide sequences were confirmed.

Analyses for mRNAs transcripted from *FGA*Δ1238 and *FGA* c.54+3C DNA fragments

Eight micrograms of each expression vector were introduced into approximately $0.5–1\times10^6$ of Chinese hamster ovary (CHO) cells in a 6-cm culture dish using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The transfected CHO cells were cultured in 5 % CO_2 at 37 °C.

The CHO cells were harvested 48 h after transfection. Total cellular RNA was extracted from cells using the QIAamp RNA Blood Mini Kit (Qiagen), and contaminated DNA was digested using the QIA shredder column and DNase, according to the manufacturer's instructions. Reverse-transcriptase (RT) reactions were carried out using oligo dT primer and RT from Moloney murine leukemia virus, followed by PCR amplification with the two pairs of primers that were used in the construction of expression vectors under the following conditions: 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C (*FGA*Δ1238) or 57 °C (*FGA* c.54+3A>C) for 1 min, and extension at 72 °C for 1 min.

The RT-PCR amplified products (cDNA) were separated by electrophoresis on 2.0 % agarose gels and purified DNA fragments were sequenced using the primers used for the construction of expression vectors. Moreover, to clarify the frequency of mRNA, the RT-PCR amplified products were inserted into pCR2.1Vector (Invitrogen, San Diego CA, USA) and transfected into INVαF' Competent Cells (Invitrogen) according to the manufacturer's instructions. Plasmids containing cDNA were purified from 15 colonies and sequenced as described above.

Haplotype analysis

The following 7 polymorphic loci neighboring the fibrinogen gene cluster on chromosome 4 were used for haplotype analysis: D4S2962, D4S2934, D4S2999, D4S3021, FGA-i3 (a TCTT repeat located in intron 3 of the *FGA*), D4S2631, and D4S1629 (ordered from centromere to telomere, according to the NCBI Map Viewer http://www.ncbi.nlm.nih.gov/projects /mapview/). The repeated regions of each short tandem repeat (STR) locus were amplified by PCR for 40 cycles using the two pairs of primers under the following conditions: denaturation at 96 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 2.5 min (**Table 1**). PCR products were diluted by distilled water and mixed with Hi-Di Formamide and GeneScan-350 ROX Size Standard. The samples were electrophoresed on an ABI Prism 310 Genetic Analyzer and fluorescent signals were analyzed with the aid of Gene Scan 3.1 software (both from Applied Biosystems).

RESULTS

Coagulation screening tests

PT, APTT, and the fibrinogen concentration, which was determined by the thrombin time method, of plasma from both Yokkaichi and Kurashiki II propositae showed a markedly prolonged clotting time or a lower concentration than the detection limit, respectively, whereas those of plasma from the parents of both propositae were within their reference ranges, indicating a normal level of fibrinogen (**Table 2**). Plasma fibrinogen concentration determined by the immunological method was 0.15 g/l for Yokkaichi and 0.028 g/l for Kurashiki II (**Table 2**).

PCR amplification and DNA sequence analysis

Amplification and sequence analysis for all exons and exon-intron boundaries in the Aα-, Bβ-, and γ-chain genes indicated the following results; no amplification product was obtained from exon 4 of the Aα-chain gene for the Yokkaichi proposita, heterozygous *FGA* c.54+3A>C (reference gene: GenBank accession # NM_000508.3) substitution was found in the Kurashiki II proposita and her mother (**Table 2**), and no abnormality was found in other regions of DNA from the two propositae and parents (data not shown).

From these results, we considered the possible large deletions in Aα-, Bβ-, and/or γ-chain genes. To amplify each entire gene, long-range PCR amplification was performed as described in Materials and methods. Although Bβ- and γ-chain genes showed wild-type base sizes (7735 and 8934 bp, respectively) for all 6 persons, the Aα-chain gene indicated aberrant small DNA (4380 bp which is 1238 bp smaller than the size of wild type, 5618 bp) in the Yokkaichi proposita and her parents, and also in the Kurashiki II proposita and her father. The Yokkaichi proposita had only aberrant small DNA and the others had both aberrant and wild-type DNA (**Fig. 1**). The nucleotide sequence of aberrant small DNA of the Aα-chain gene was determined by direct sequence analysis. The sequence showed a large deletion of *FGA* (c.364+86_510+43del1238) (reference coding DNA: GenBank accession # NM_000508.3) for the Yokkaichi propositae, her parents, the Kurashiki II propositae, and her father (**Table 2**). Genomic DNA was deposited as GenBank accession #M64982.1 [10].

Analysis of fibrinogen Aα-chain gene transcripts in CHO cells

To verify whether the DNA mutation of *FGA*Δ1238 and *FGA* c.54+3C influences the transcription of mature mRNAs, mutant Aα-chain mRNAs were transiently produced in CHO cells and analyzed as described in Materials and methods. The RT-PCR products from CHO cells transfected with pT-Aα-Mt1 showed two bands, which were named Aα-M1-1 and Aα-M1-2, respectively **(Fig. 2A**). Direct sequencing demonstrated that the mRNA of Aα-M1-1 was a combined product of exon 3, intron 3, intron 4, and exon 5, whereas the mRNA of Aα-M1-2 was a splicing product of exon 3 and exon 5 (**Fig. 2A**). The frequency of the mRNA of Aα-M1-1 and Aα-M1-2 was 14 % (2/14) and 86 % (12/14), respectively.

The RT-PCR products from CHO cells transfected with pT-Aα-Wt2 showed a single band that was named Aα-W2, and those with pT-Aα-Mt2 showed two bands, which were named Aα-M2-1 and Aα-M2-2, respectively (**Fig. 2B**). Direct sequencing showed that Aα-W2 had normal mRNA with correct splicing of exon 1 and exon 2. The mRNA of Aα-M2-1 was a combined product of exon 1, intron 1, and exon 2, whereas the mRNA of Aα-M2-2 included two products, one (Aα-M2-2A) was a normal splicing product of exon 1 and exon 2, and the other (Aα-M2-2B) was an aberrant splicing product of exon 1 and exon 2 using a cryptic 5' splice site 18-nt upstream from the end of exon 1 (**Fig. 2B**). The frequency of the mRNA of Aα-M2-1, Aα-M2-2A and Aα-M2-2B was 46 % (6/13), 8% (1/13), and 46 % (6/13), respectively.

Haplotype analysis of chromosome 4q adjacent to fibrinogen gene

Haplotype analysis (D4S2999, D4S3021, FGA-i3, and D4S2631) revealed that

deletions of *FGA*Δ1238 were present on three different chromosomes, with haplotypes 217-234-del-212, 217-232-del-204, and 207-232-del-212 (**Fig. 3**). On the other hand, *FGA* c.54+3C mutation was present on the chromosome with haplotypes 215-232-199-208 **(Fig. 3**).

DISCUSSION

In this study, we described two afibrinogenemias, Fibrinogen Yokkaichi and Kurashiki II, each caused by a homozygote of *FGA*Δ1238 and a compound heterozygote of *FGA*Δ1238 and *FGA* c.54+3C, respectively. The six families, including our two cases, with *FGA*Δ1238 mutation have only been found in Far Eastern countries, one in China [11] and five in Japan [12], (E Hamada, T Sawamura, N Yokota, M Maekawa: abstract P13-05, 54th Annual Meeting of the Japanese Society of Laboratory Medicine, Nov. 2007, in Japanese) and (T Sawamura, E Hamada, N Kanayama, M Maekawa: abstract O-171, 55th Annual Meeting of the Japanese Society of Laboratory Medicine, Nov. 2008, in Japanese). Although *FGA* c.54+3A>C substitution is a novel mutation, A>G substitution at an identical site (*FGA* c.54+3A>G) has been reported in France [13].

To examine the causes of the low level of plasma fibrinogen in individuals with *FGA*Δ1238 mutation or *FGA* c.54+3A>C substitution, we analyzed the mRNAs transcribed from the cloned mutant Aα-chain genes in CHO cells. A minigene corresponding to *FGA*Δ1238, which included the region between intron 2 and exon 5, generated two mRNAs. One was a non-spliced product and the other was a spliced product connecting exon 3 and 5, both of which induced a frameshift of coding amino

acids and finally terminated at premature termination codon 114 and 106, respectively. On the other hand, a minigene corresponding to *FGA* c.54+3C, which includes nucleotides between the 5'-flanking region and intron 2, generated three mRNAs. One is a normal splicing product but with only 8 % Aα-chain mRNA. The others are a non-splicing product and an aberrant splicing product. The former might induce a frameshift of coding amino acids and finally terminate at premature termination codon 31. The latter uses the cryptic 5' splice site in exon 1, resulting in deletion of 6 amino acids (-7– -2; VVGTAW) of signal peptides of the Aα-chain composed of 19 amino acids (-19– -1). Overall, the above-mentioned mRNAs generating the premature termination codon might be degraded by the nonsense-mediated mRNA decay system, causing a deficiency of intact Aα protein, whereas the mature Aα-chain having aberrant signal peptides might lead to impaired secretion because signal peptides play a crucial role in the transportation of protein in the cell and secretion of the protein outside the cell [13, 14].

In Europe and the United States of America, a 11 kb deletion of the Aα-chain gene and *FGA* c.510+1G>T substitution have been reported as afibrinogenemia in 8 and 14 families, respectively (fibrinogen variant database). Furthermore, haplotype analysis indicates that each mutation occurred on several different ancestral chromosomes [13, 16, 17]. In particular, the 11 kb deletion of the Aα-chain gene was caused by non-homologous (illegitimate) recombination [18], whereas the 6 families with the *FGA*Δ1238 mutation were found only in Far Eastern countries, and our haplotype analysis indicated the possibility that the 3 mutation genes might be derived from three different origins, namely, nucleotide deletion events occurred on 3 different ancestral

chromosomes. We also speculate that the *FGA*Δ1238 mutation might arise from non-homologous recombination similar to the 11 kb deletion of the Aα-chain gene.

Although the parents of both propositae were heterozygous for fibrinogen Aα-chain gene mutation of *FGA*Δ1238 or *FGA* c.54+3C, their plasma fibrinogen levels were each in the normal range. This was unusual because both *FGA*Δ1238 and *FGA* c.54+3C mutations are the non-synthesizing type of fibrinogen Aα-chain, suggesting that heterozygotes have a lower level of fibrinogen (1/2–1/4 of wild type). Afibrinogenemias caused by splicing abnormalities have been reported as homozygous mutations, and there is a report that a heterozygous splice site mutation of the Aα-chain gene does not cause hypofibrinogenemia [19]. We considered that the plasma fibrinogen level might be regulated by the expression level of Bβ- and/or γ-chain. Previous studies of fibrinogen synthesis with Bβ-chain cDNA in HepG2 or COS-1 cells have shown that an excess amount of Aα- and γ-chains accumulated in the cells [20-23], suggesting that the synthesis of the Bβ-chain is a rate-limiting peptide for the assembly and secretion of mature fibrinogen. Finally, we speculated that the normal level of plasma fibrinogen in the Yokkaichi and Kurashiki II parents was caused as follows: synthesis of an excess amount of normal Aα-chain mRNA (and peptide) from the normal allele compensates for aberrant Aα-chain mRNA (and peptide) or a reduced level of Aα-chain mRNA (and peptide) from the mutant allele.

In conclusion, we report two cases of afibrinogenemia, Yokkaichi and Kurashiki II, caused by homozygous *FGA*Δ1238 and a compound heterozygous for *FGA*Δ1238 and *FGA* c.54+3C, respectively. Molecular analyses of both genetic variants suggested that the lack of a mature Aα-chain, impaired assembly and/or secretion of fibrinogen

molecule might lead to afibrinogenemia.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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AUTHORSHIP

Y. Takezawa performed research, analyzed data, and wrote the paper,

F. Terasawa and K. Matsuda performed research and analyzed data.

M. Sugano and N. Okumura designed the research and reviewed the paper.

A. Tanaka, M. Fujiwara, and K. Kainuma collected the samples and designed the research.

References

[1] Doolittle RF, Bouma IH, Cottrell BA, Strong D, Watt KWK. The covalent structure of human fibrinogen. In: Bing DH, editor. The chemistry and physiology of the human plasma proteins. Oxford: Pergamon Press; 1979. p.77-95.

[2] Doolittle RF. Fibrinogen and fibrin. Sci Amer. 1981; 245: 92-101.

[3] Kant JA, Fornace AJ, Saxe D, Simon MI, McBride OW, Crabtree GR. Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion. Proc Natl Acad Sci USA. 1985; 82: 2344-8.

[4] de Moerloose P, Neerman-Arbez M. Congenital fibrinogen disorders. Semin Thromb Hemost. 2009; 35: 356-66.

[5] Acharya SS, Dimichele MD. Rare inherited disorders of fibrinogen. Haemophilia. 2008; 14: 1151-8.

[6] Asselta R, Duga S, Tenchini ML. The molecular basis of quantitative fibrinogen disorders. J Thromb Haemost. 2006; 4: 2115-29.

[7] Vu D, Neerman-Arbez M. Molecular mechanisms accounting for fibrinogen deficiency: from large deletions to intracellular retention of misfolded proteins. J Thromb Haemost. 2007; 5: 125-31.

[8] Neerman-Arbez M, de Moerloose P. Mutations in the fibrinogen gene cluster accounting for congenital afibrinogenemia: an update and report of 10 novel mutations. Hum Mutat. 2007; 28: 540-53.

[9] Terasawa F, Kamijyo Y, Fujihara N, Yamauchi K, Kumagai T, Honda H, et al. *In vitro* transcription of compound heterozygous hypofibrinogenemia Matsumoto IX; first identification of *FGB* IVS6 deletion of 4 nucleotides and *FGG* IVS3-2A>C

causing abnormal RNA splicing. Clin Chim Acta. 2010; 411: 1325-9.

[10] Chung DW, Harris JE, Davie EW. Nucleotide sequences of the three genes coding for human fibrinogen. In: Chien S, editor. Fibrinogen, thrombosis, coagulation and fibrinolysis. New York: Plenum Press; 1991. p.39-48.

[11] Fang Y, Dai BT, Wang XF, Fu QH, Dai J, Xie F, et al. Identification of three FGA mutations in two Chinese families with congenital afibrinogenaemia. Haemophilia. 2006; 12: 615-20.

[12] Watanabe K, Shibuya A, Ishii E, Kurihara M, Inoue S, Ono M, et al. Identification of simultaneous mutation of fibrinogen a chain and protein C genes in a Japanese kindred. Br J Haematol. 2003; 120: 101-8.

[13] Neerman-Arbez M, de Moerloose P, Bridel C, Honsberger A, Schönbörner A, Rossier C, et al. Mutations in the fibrinogen Aα gene account for the majority of cases of congenital afibrinogenemia. Blood. 2000; 96: 149-52.

[14] Isken O, Maquat LE. Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. Genes Dev. 2007; 21: 1833-56.

[15] Doma MK, Parker R. RNA quality control in eukaryotes. Cell. 2007; 131: 660-8.

[16] Neerman-Arbez M, Honsberger A, Antonarakis SE, Morris MA. Deletion of the fibrinogen alpha-chain gene (*FGA*) causes congenital afibrinogenemia. J Clin Invest. 1999; 103: 215-8.

[17] Neerman-Arbez M, de Moerloose P, Honsberger A, Parlier G, Arnuti B, Biron C, et al. Molecular analysis of the fibrinogen gene cluster in 16 patients with congenital afibrinogenemia: novel truncating mutations in the *FGA* and *FGG* genes. Hum Genet. 2001; 108: 237-40.

[18] Neerman-Arbez M, Antonarakis SE, Honsberger A, Morris MA. The 11 kb *FGA* deletion responsible for congenital afibrinogenaemia is mediated by a short direct repeat in the fibrinogen gene cluster. Eur J Hum Genet. 1999; 7: 897-902.

[19] Polack B, Pouzol P, de Moerloose P, Gay V, Hanss M. Is primary prophylaxis required in afibrinogenemia? Transfusion. 2010; 50: 1401-3.

[20] Angles-Cano E, Mathonnet F, Dreyfus M, Claeyssens S, de Mazancourt P. A case of afibrinogenemia associated with A-alpha chain gene compound heterozygosity (HUMFIBRA c.[4110delA]+[3200+1G>T]). Blood Coagul Fibrinolysis. 2007; 18: 73-5.

[21] Yu S, Sher B, Kudryk B, Redman CM. Fibrinogen precursors. Order of assembly of fibrinogen chains. J Biol Chem. 1984; 259: 10574 -81.

[22] Roy SN, Mukhopadhyay G, Redman CM. Regulation of fibrinogen assembly. Transfection of HepG2 cells with Bβ cDNA specifically enhances synthesis of the three component chains of fibrinogen. J Biol Chem. 1990; 265: 6389-93.

[23] Roy S, Overton O, Redman C. Overexpression of any fibrinogen chain by HepG2 cells specifically elevates the expression of the other two chains. J Biol Chem. 1994; 269: 691-5.

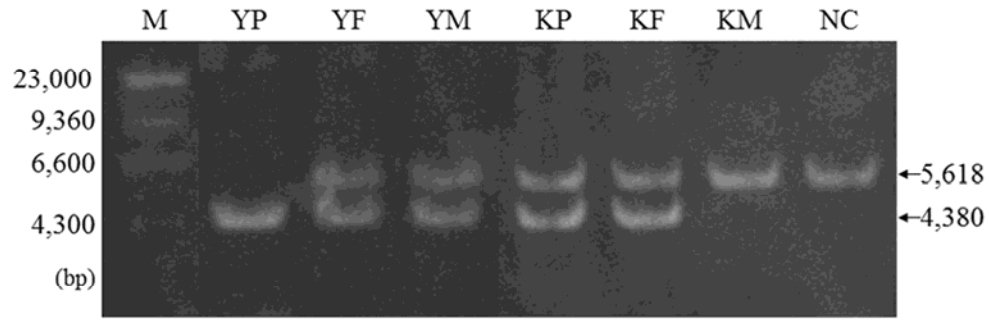

Figure 1. Detection of Aα-chain gene deletion by long-range PCR amplification.

Long-range PCR detected the deletion of the fibrinogen Aα-chain gene. Normal control subject showed a 5618 bp PCR product, whereas the Yokkaichi proposita, her parents, the Kurashiki II proposita, and her father showed shorter products (4380 bp). M: DNA size marker, YP: Yokkaichi proposita, YF: Yokkaichi father, YM: Yokkaichi mother, KP: Kurashiki II proposita, KF: Kurashiki II father, KM: Kurashiki II mother, NC: Normal control.

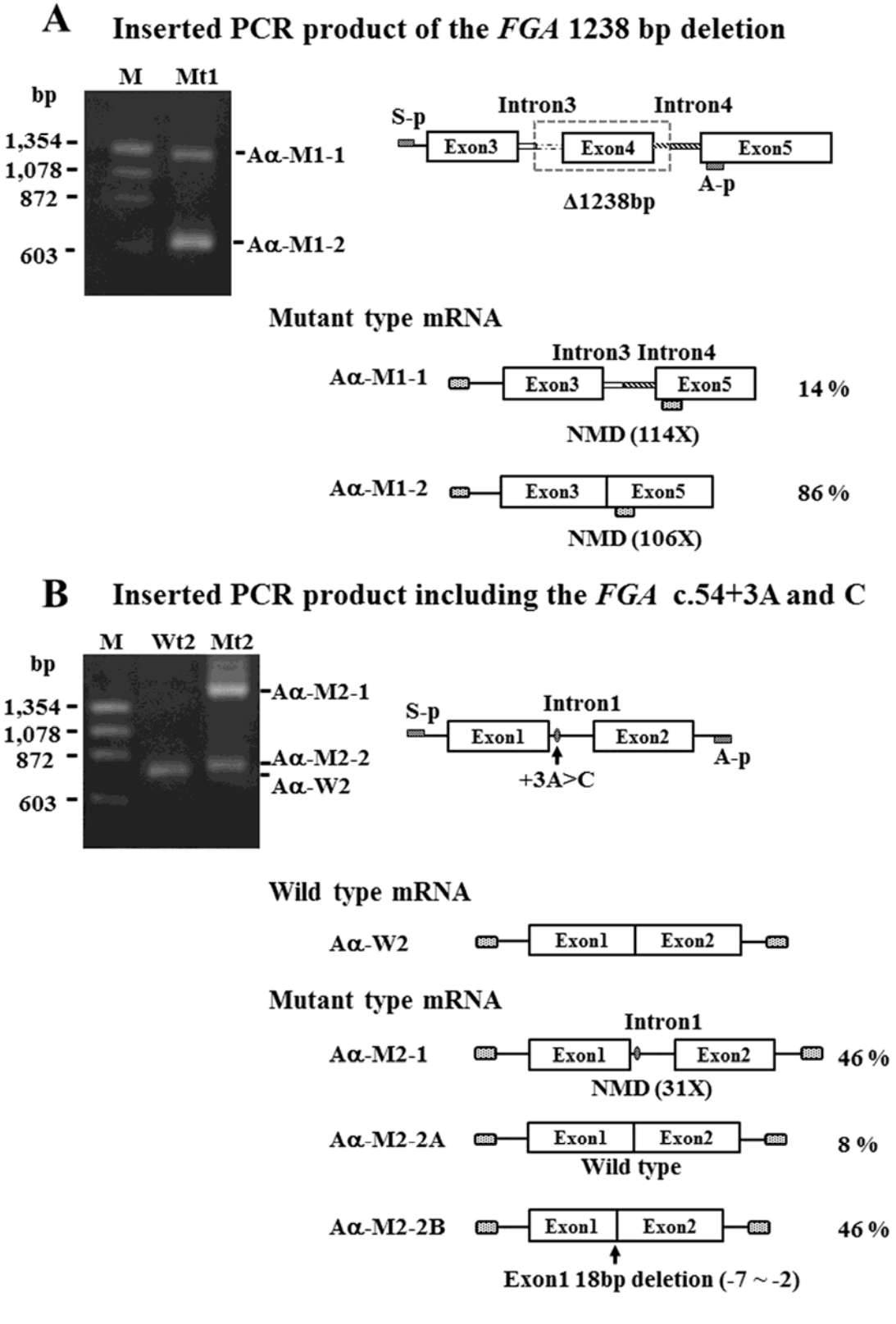
A
Inserted PCR product of the FGA 1238 bp deletion
M
Mt1
bp
1,354
1,078
872
603
Aα-M1-1
Aα-M1-2
S-p
Intron3
Intron4
Exon3
Exon4
Exon5
A-p
Δ1238bp
Mutant type mRNA
Aα-M1-1
Intron3 Intron4
Exon3
Exon5
14 %
NMD (114X)
Aα-M1-2
Exon3
Exon5
86 %
NMD (106X)
B
Inserted PCR product including the FGA c.54+3A and C
M
Wt2
Mt2
bp
1,354
1,078
872
603
Aα-M2-1
Aα-M2-2
Aα-W2
S-p
Intron1
Exon1
Exon2
A-p
+3A>C
Wild type mRNA
Aα-W2
Exon1
Exon2
Mutant type mRNA
Intron1
Aα-M2-1
Exon1
Exon2
46 %
NMD (31X)
Aα-M2-2A
Exon1
Exon2
8 %
Wild type
Aα-M2-2B
Exon1
Exon2
46 %
Exon1 18bp deletion (-7 ~ -2)

Figure 2. Analysis of fibrinogen Aα-chain minigene transcripts in CHO cells.

PCR-amplified minigenes of the Kurashiki II proposita were cloned into pTARGET vector. Panel (A,B) left: RT-PCR products separated on a 2 % agarose gel. Panel (A,B) upper right: PCR product amplified with sense (S-p) and antisense primers (A-p). Panel (A,B) lower right: Schematic mRNAs predicted from wild-type and mutant-type genes.

Panel (A) is a minigene from *FGA* intron 2 to exon 5 including *FGA* 1238 bp deletion. Lane M: DNA size marker, Mt1: RT-PCR products amplified from mutant mRNA of the *FGA* 1238bp deletion. Aα-M1-1 is an aberrant mRNA, which is the combined product of exon 3, intron 3, intron 4, and exon 5. Aα-M1-2 is an aberrant mRNA with splicing products of exon 3 and exon 5. The frequency of the mRNA of Aα-M1-1 and Aα-M1-2 was 14 % and 86 %, respectively.

Panel (B) is a minigene from the *FGA* 5'-flanking region to intron 2 including *FGA* c.54+3A or C. Wt2: RT-PCR products amplified from wild-type mRNA of *FGA* c.54+3A, and Mt2: RT-PCR products amplified from mutant mRNA of *FGA* c.54+3C. Aα-W2 is normal mRNA from the wild-type *FGA* c.54+3A gene. Aα-M2-1 is an aberrant mRNA, which is the combined product of exon 1, intron 1, and exon 2. Aα-M2-2A is a normal splicing product of exon 1 and exon 2. Aα-M2-2B is an aberrant splicing product of exon 1 and exon 2, in which 18-nt (upstream of the end of exon 1) was deleted. The frequency of the mRNA of Aα-M2-1, Aα-M2-2A and Aα-M2-2B was 46 %, 8%, and 46 %, respectively.

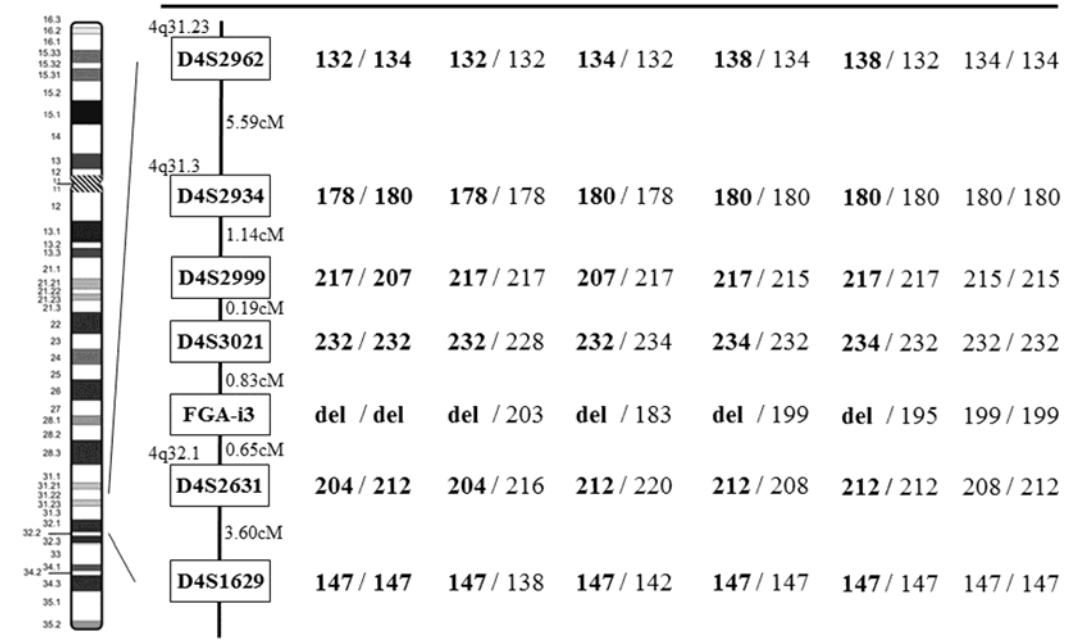

Figure 3. Haplotype analysis of chromosome 4q adjacent to fibrinogen gene.

Chromosomal localization of markers used for haplotype analysis is shown on the ideogram of chromosome 4 (on the left). Haplotypes of Yokkaichi and Kurashiki II family members are shown as nucleotide numbers of PCR amplified products (on the right).

Table1. Oligonucleotide sequence of each primer for STR-PCR.

Marker		Oligonucleotide (5'→3')	Size of PCR product (bp)
D4S2962	sense	FAM-TAGCAGGCTATCTCTCAATACAC	127-143
	antisense	ACTTGGCTAGTCAAGCACAC	
D4S2934	sense	FAM-CAAAACAGATCAGGATGTGG	155-189
	antisense	TTGCTGTCTTTACAGAGCACC	
D4S2999	sense	FAM-GTTTGTTGCCTGAATTTCC	178-218
	antisense	CATGTCCATTTCCTCAAGTC	
D4S3021	sense	FAM-ACTGGCCTGATGTGGTGA	223-245
	antisense	GGTGCCTGATAGCCTGAA	
FGA-i3	sense	FAM-GCCCCATAGGTTTTGAACTCA	196-240
	antisense	TGATTTGTCTGTAATTGCCAGC	
D4S2631	sense	FAM-TTCAATACTCCTGTATCACAAAGG	202-222
	antisense	TGAGACACAATCTGAGCTATGC	
D4S1629	sense	FAM-TGGTTCTGCTTTTTCTCTCC	141-157
	antisense	TTTAACAGACAAATGACAAATCTG	

FAM: Fluorescein amidite

Table 2. Results of coagulation screening tests and nucleotide sequence of the fibrinogen gene

Family	PT (sec.)	APTT (sec.)	Fbg (g/l) Thrombin-time method	Fbg (g/l) Immunological method	Mutation of DNA	Genotype
(Reference interva	10.8-13.2	23.0-38.0	1.80-3.50	1.80-3.50		
Yokkaichi						
Proposita	> 60.0	> 200.0	<0.20	0.15*	*FGA* Δ1238 bp	Homozygote
Father	11.3	29.0	1.91	2.48*	*FGA* Δ1238 bp	Heterozygote
Mother	11.8	29.0	1.91	2.92*	*FGA* Δ1238 bp	Heterozygote
Kurashiki II						
Proposita	> 60.0	> 200.0	<0.20	0.028	*FGA* c.54+3A>C *FGA* Δ1238 bp	Compound heterozygot
Father	11.7	28.6	3.13	3.11	*FGA* Δ1238 bp	Heterozygote
Mother	12.4	29.3	2.23	2.31	*FGA* c.54+3A>C	Heterozygote

* EDTA plasma

PT: prothrombin time, APTT: activated partial thromboplastin time, Fbg: fibrinogen