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Brief and descriptive title: Down-regulation of Fbg gene by siRNA

siRNA down-regulation of *FGA* mRNA in HepG2 cells demonstrated that heterozygous abnormality of the Aα-chain gene does not affect the plasma fibrinogen level

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Abstract

Introduction: We encountered two afibrinogenemia patients with homozygous and compound heterozygous *FGA* mutation. Of interest, the patients' parents who are heterozygous, had normal levels of plasma fibrinogen; thus, we hypothesized that liver *FGA* mRNA levels were higher than those of *FGB* and/or *FGG* mRNA. *Materials and Methods*: To examine the hypothesis, we quantitated mRNA levels of a normal liver and human hepatocyte cell line, HepG2 cells, and performed siRNA-mediated down-regulation of the fibrinogen gene in HepG2 cells. mRNA levels were determined using real-time quantitative RT- PCR for three normal livers and HepG2 cells. Down-regulation of *FGA*, *FGB*, or *FGG* in HepG2 cells was performed by the addition of siRNA corresponding to each of the three genes, and the mRNA levels determined in the cells and the secreted fibrinogen concentration in media. *Results*: mRNA level of normal human liver was *FGA* = *FGB* > *FGG* and *FGG* mRNA level was about 2-fold lower than the others, that of HepG2 cells was *FGA* > *FGG* > *FGB* and *FGA* mRNA was approximately 2- or 4-fold higher than *FGG* mRNA and *FGB* mRNA. When *FGA*, *FGB*, or *FGG* mRNA expression levels were down-regulated by nearby 50%, fibrinogen concentrations in media were 78 %, 49 %, or 57 % of control, respectively. *Conclusions*; Our results suggest that *FGG* mRNA levels are limiting fibrinogen expression in normal liver and HepG2 cells and that 50% reduction of *FGA* mRNA levels would not limit fibrinogen expression in normal liver and HepG2 cells.

Keywords: afibrinogenemia, heterozygote, Aα-chain, mRNA, siRNA, real-time quantitative RT- PCR

Abbreviations: *FGA*, fibrinogen alpha; *FGB*, fibrinogen beta; *FGG*, fibrinogen gamma; *FGA*Δ1238, deletion of 1238 bases of the Aα-chain gene; NMD, nonsense-mediated mRNA decay; siRNA, short interfering RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; *ABL*, *abelson*; FAM, fluorescein amid; BHQ1, black hole quencher 1; TAMRA, tetramethylrhodamine.

Introduction

Fibrinogen is a 340 kDa plasma glycoprotein composed of two sets of three different polypeptide chains (Aα, Bβ, and γ) and 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 residues, which comprise 3 genes coding for fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*), and fibrinogen gamma (*FGG*), clustered in a region of approximately 50 kb on chromosome 4q31.3. The 3 genes contain the following numbers of exons: 6 (included Aα-E isoform) for *FGA*, 8 for *FGB* and 10 for *FGG* [3-5]. Genetic mutations in fibrinogen chain genes have been associated with afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia, as listed in the fibrinogen variant database [6].

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 [4-9]. In particular, reports causing large deletions between 1.2 and 15 kb were restricted in *FGA* [6]. 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 Yokkaichi and Kurashiki II, respectively [10].

Two probands showed homozygous abnormality of *FGA* but, as in the patients' parents, the fibrinogen activity and antigen were normal in spite of having a heterozygous genetic abnormality. Six families, including our two cases, with *FGA*Δ1238 have only been found in Far Eastern countries, one in China [11] and five in Japan [10, 12-14], and regarding which parents and family members have a heterozygous genetic abnormality, the plasma fibrinogen

concentrations were all normal (Table 1). According to our observation and the coincident reports, we hypothesize these phenomena as follows. When patients have a genetic mutation in *FGA* causing large deletion, nonsense, frameshift, or splicing abnormality etc., all of which lead to premature termination and nonsense-mediated mRNA decay (NMD), mRNA levels in heterozygous patients' hepatocytes were reduced by approximately half. Under these conditions, if *FGA* mRNA was more than two-fold higher than that of *FGB* and/or *FGG* mRNA, fibrinogen production (assembly and secretion) in hepatocytes and plasma fibrinogen level was not decreased by around half.

In this study, we transfected short interfering RNA (siRNA) of each fibrinogen gene into a human hepatocyte cell line, HepG2 cells, which produce fibrinogen in culture media. We also compared the associations between mRNA levels of *FGA*, *FGB*, or *FGG* in HepG2 cells and fibrinogen concentration in media. Further, we bibliographically compared the plasma fibrinogen levels of the patients with heterozygous mutations in *FGA*, *FGB*, or *FGG* genes.

Materials and methods

Cell culture

A human hepatocyte cell line, HepG2 cells, was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin (GIBCO) in a 5% CO_2 incubator at 37°C.

Fibrinogen gene expression in normal human liver cells

Total RNA from normal human livers (liver RNA #1: 60-year-old man, liver RNA #2: 64-year-old man, liver RNA #3: 86-year-old man) was purchased from BioChain (Newark,

CA, USA). The cDNA was synthesized from 2 μg total RNA in 40μl reaction buffer and analyzed as described below by real-time quantitative RT- PCR.

siRNA-mediated down-regulation of the fibrinogen gene, FGA, FGB, or FGG, in HepG2 cells

Silencer Select Pre-designed *FGA* siRNA (catalog no. 4392420, IDs5115), *FGB* siRNA (ID s5119), *FGG* siRNA (ID s5179), and Negative Control #1 siRNA (catalog no. 4390843) were obtained from Ambion (Austin, TX, USA). Each siRNA was reversely transfected into HepG2 cells in technical triplicates in 3 independent experiments. Briefly, each siRNA was mixed with 100 μl OPTI-MEM I Reduced Serum Medium (GIBCO) in a 24-well tissue culture plate, and 1 μl of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) was added to each well, mixed gently, and incubated for 20 minutes at room temperature according to the manufacturer's protocol. Then, 500 μl HepG2 cells suspension containing 5 x 10^4 cells was added to the mixture of siRNA reagent. The final concentrations of *FGA, FGB,* and *FGG* siRNA varied between 1.0 and 30 nM and negative control siRNA was used at 10 nM, and the absence of siRNA was used as a non-transfected control. To ensure the siRNA effects on fibrinogen production, 6 hours after transfection, medium was removed, and 600 μl fresh antibiotic-free medium were added to each well. Thirty hours after transfection, culture medium was collected and fibrinogen concentrations determined by enzyme-linked immunosorbent assay (ELISA). HepG2 cells were trypsinized and harvested to perform quantitative analysis of each mRNA. To obtain a nearly 50 % decrease of *FGA, FGB,* or *FGG* mRNA expression in each experiment, siRNA concentrations varied from 1.0 to 2.0 nM.

RNA extraction and synthesis of complementary DNA (cDNA)

Total RNA was extracted from siRNA transfected HepG2 cells using a QIAamp RNA blood mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The cDNA was synthesized from 20 μl total RNA solution in 40 μl reaction buffer containing 1 mmol/l of each deoxynucleotide triphosphate (Invitrogen), 1 mmol/l dithiothreitol (Invitrogen), 1.26 μmol/l random hexamer primers (Promega, Madison, WI, USA), and 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reverse transcription (RT) reaction was performed at 42 °C for 1 h.

Real-time quantitative RT- PCR

Each cDNA was amplified in triplicate in a 96-well plate and normalized to the expression of *ABL* (*abelson*) as an endogenous reference control. The reaction mixture contained 2 μl cDNA, 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA), 0.5 μmol/l of each primer, and 0.25 μmol/l TaqMan probe in a total of 20 μl. Primers and TaqMan probes, including fluorescein amid (FAM) at the 5'-end nucleotide and a quencher (black hole quencher 1,BHQ1 or tetramethylrhodamine, TAMRA) at the 3'-end nucleotide, were as follows: *FGA*: forward primer (5'-GGATCGTCTGCCTGGTCCTA-3'), reverse primer (5'-CCTTCAGCTAGAAAGTCACCTTCA-3'), and probe (5'-FAM-TGCAGTCCATGCTGTGCCCACCAC-BHQ1-3'), *FGB*: forward primer (5'-GTCCCAAGGTGTCAACGACAA-3'), reverse primer (5'-CTCTCTTCTTGTCAAGGGGTCG-3'), and probe (5'-FAM-ACCACGGGCACTGAAGAAACCCTCCT-BHQ1-3'), *FGG*: forward primer (5'-CAGGATGGATCTGGTTGGTGG-3'), reverse primer (5'-GGAGTAGATGCTTTTGAGTAAGTGC-3'), and probe (5'-FAM-TGAACAAGTGTCACGCTGGCCATCTCAA-BHQ1-3'), *ABL*: forward primer

(5’-TGGAGATAACACTCTAAGCATAACTAAAGGT-3’), reverse primer (5’-GATGTAGTTGCTTGGGACCCA-3’), and probe (5’-FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA-3’).

Real-time quantitative RT-PCR was performed using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) at 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. Threshold cycle (Ct) values were obtained using SDS, Version 2.3 software (Applied Biosystems). To determine the relative mRNA levels between target and endogenous reference control mRNA, the comparative Ct ($2^{-\Delta\Delta Ct}$) method was used. Briefly, the expression of the target gene was calculated as follows: $\Delta\Delta Ct = (Ct_{target} - Ct_{ABL})_{siRNA\ x} - (Ct_{target} - Ct_{ABL})_{siRNA\ 0}$ (siRNAx means objective siRNA concentration)[15].

Validation of the $2^{-\Delta\Delta Ct}$ method

Validation assays for the efficiency of amplification of the target gene (*FGA*, *FGB* and *FGG*) and endogenous reference control gene (*ABL*) were examined using real-time PCR and TaqMan detection. The cDNA was synthesized from HepG2 cells, and serial dilutions of cDNA were amplified by real-time PCR, as described in Materials and Methods. The average Ct was calculated for the target gene (Ct_{target}) and *ABL* (Ct_{ABL}), and the ΔCt ($Ct_{target} - Ct_{ABL}$) was determined. The log cDNA dilution was plotted against ΔCt.

ELISA assay

To determine the fibrinogen concentrations of culture media, ELISA was performed using the following reagents: capture antibody: goat anti-human fibrinogen antibody (Cappel, Durham, NC) (1:1,000 dilution), secondary antibody: goat anti-human fibrinogen antibody (Abcam,

Tokyo, Japan) biotinated using Biotin labeling Kit-NH_2 (Dojindo Laboratories, Mashiki-cho, Kumamoto, Japan) (1:40,000 dilution), detection reagents: streptoavidin conjugated horseradish peroxidase (Beckman Coulter, Fullerton, CA, USA) (1:8,000 dilution) and TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Calibration curve for fibrinogen was linear over a concentration range of 20-200 ng/ml and the limit of detection was 5 ng/ml.

Statistical analysis

The data were derived from repeated experiments for each assay and values are expressed as the mean ± SD. Datasets were analyzed using Student's *t* test and $p < 0.05$ was considered significant.

Results

Fbg mRNA expression for human normal liver and HepG2 cells

Real-time quantitative RT- PCR for fibrinogen mRNA was performed for normal human liver RNA and HepG2 cells, as described in Materials and Methods. Representative amplification curves of mRNA of *FGA*, *FGB*, and *FGG* are shown in Figure 1. The threshold cycle, Ct, for three normal human liver RNA in each triplicate experiments was as follows: liver RNA#1: *FGA*: 15.95 ± 0.036, *FGB*: 15.98 ± 0.028, *FGG*: 16.90 ± 0.078 (Figure 1A), liver RNA#2: *FGA*: 17.35 ± 0.053, *FGB*: 17.01 ± 0.025, *FGG*: 18.85 ± 0.097, liver RNA#3: *FGA*: 15.33 ± 0.030, *FGB*: 15.02 ± 0.052, *FGG*: 16.71 ± 0.024. Since mRNA expression levels varied among individuals, ΔCt was calculated on the basis of *FGA* in each person. Consequently, average ΔCt was as follows: $\Delta Ct_{FGB\text{-}FGA}$: -0.21 ± 0.19, $\Delta Ct_{FGG\text{-}FGA}$: 1.28 ± 0.26. Expression levels of *FGA* and *FGB* mRNA were approximately equal, but that of *FGG*

mRNA was about 2-fold lower than the others. On the other hand, the Ct for HepG2 cells was *FGA*: 18.85 ± 0.16, *FGB*: 20.63 ± 0.18, and *FGG*: 19.62 ± 0.22 (Figure.1B). In brief, *FGA* mRNA was transcribed approximately 2- or 4-fold more than *FGG* mRNA or *FGB* mRNA.

Validation of the $2^{-\Delta\Delta Ct}$ method

The $2^{-\Delta\Delta Ct}$ method can be used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. The slope of the line was as follows: *FGA*: 0.062, *FGB*: 0.100, *FGG*: 0.082. Absolute value of the slope was 0.1or less, indicating that the amplification efficiencies of the target and endogenous reference control genes are approximately equal; therefore, the ΔΔCt calculation for relative quantification of the target can be used to analyze the data [15].

siRNA down-regulation of fibrinogen gene on HepG2 cells

To determine the siRNA concentration that could inhibit the mRNA expression level by nearby 50 %, we varied the concentration of each siRNA (between 1.25 and 30.0 nM) added to culture media. Each mRNA level was determined by real-time quantitative RT-PCR, and each fibrinogen concentration in the culture media was determined by ELISA. As shown in Figure 2, mRNA levels of the 3 genes and the fibrinogen concentration decreased in a dose-dependent manner with the increased concentration of siRNA. With the addition of 1.5 nM *FGA* siRNA, the relative amount of the *FGA* mRNA was 41.5 % and the fibrinogen concentration was 76.2 % versus siRNA 0 concentration (both mRNA and fibrinogen levels were 100 %), whereas, with the addition of 1.5 nM *FGB* siRNA, the relative amount of *FGB* mRNA was 55.5 % and the fibrinogen concentration was 53.0 % versus siRNA 0

concentration, and with the addition of 1.25 nM *FGG* siRNA, the relative amount of *FGG* mRNA was 46.5 % and the fibrinogen concentration was 53.4 % versus siRNA 0 concentration.

Association between down-regulation of fibrinogen mRNA and fibrinogen production

An experiment was performed to inhibit 50 % mRNA expression as a model having heterozygous abnormality, in which mRNA levels are lower than normal as a result of a large deletion, nonsense, frame shift mutation, or splicing abnormality. According to the results shown in Figure 2, to obtain nearby 50 % inhibition of mRNA expression, siRNA concentrations were 1.0, 1.25, 1.5, and 2.0 nM in each independent experiment. Almost 50 % inhibition of mRNA expression was obtained with 1.25 or 1.5 nM for *FGA* siRNA, 1.5 nM for *FGB* siRNA, and 1.0 or 1.25 nM for *FGG* siRNA. The mean mRNA expression levels in 3 independent down-regulation experiments were reduced to 46.3 ± 4.4 % for *FGA*, 53.8 ± 1.6 % for *FGB*, and 45.2 ± 1.3 % for *FGG* as compared with 100 % mRNA under the condition of siRNA 0 concentration (non-transfected control) (Figure 3). *FGB* and *FGG* mRNA levels were not significantly different from *FGA* mRNA level ($p > 0.05$). When almost 50% inhibition of each mRNA expression was obtained, protein concentrations of fibrinogen were 77.7 ± 7.1 % for *FGA*, 48.7 ± 3.8 % for *FGB*, and 56.7 ± 3.0 % for *FGG*, respectively (Figure 3). Fibrinogen production when *FGA* mRNA was down regulated to 46.3 % was significantly higher level to $p = 0.032$ versus 53.8 % of *FGB* mRNA or $p = 0.027$ versus 45.2 % of *FGG* mRNA, respectively. Under the condition of siRNA 0 concentration, fibrinogen concentration in the 3 independent experiments was 84.2 ng/ml, 144.2 ng/ml, and 157.7 ng/ml, respectively, whereas, with the addition of the siRNA negative control, the production of the 3 mRNA and fibrinogen was not reduced. Further, 3 mRNA levels and

fibrinogen concentrations in the siRNA negative control were not significantly different from each other ($p > 0.05$).

Discussion

We encountered two afibrinogenemic patients' parents who are heterozygous genetic *FGA* abnormality and normal levels of plasma fibrinogen [10]. Thus, we hypothesized that in normal liver, *FGA* mRNA was more than two-fold higher than *FGB* and/or *FGG* mRNA, and if *FGA* mRNA was decreased by almost half by a mutation with *FGA* mRNA decay, plasma fibrinogen was not decreased and was maintained in the normal range. To examine the hypothesis, we quantitated mRNA levels for three normal livers and a human hepatocyte cell line, HepG2 cells, and performed siRNA-mediated down-regulation of fibrinogen genes, *FGA*, *FGB*, and *FGG*, in HepG2 cells.

Real-time quantitative RT-PCR showed that mRNA level in the normal human liver was *FGA* = *FGB* > *FGG*, and the *FGG* mRNA level was about 2-fold lower than the others, that of HepG2 cells was *FGA* > *FGG* > *FGB*, and *FGA* mRNA was approximately 2- or 4-fold higher than *FGG* mRNA or *FGB* mRNA. Result obtained from the normal human liver implies that *FGG* mRNA levels are limiting fibrinogen production in normal liver. We thought that the discordancy in the order of mRNA levels between in the normal human liver and in HepG2 cells might be caused by somatic changes in tumor cells. Yu et al. performed a pulse experiment on HepG2 cells using L-[^{35}S] methionine and demonstrated that the rate of synthesis of the three component chains of fibrinogen is unequal to that of the Bβ chain, being less than that of the Aα and γ chains, and the rate was faster in the order Aα, γ, and Bβ chain [16]. This order coincided with our order of mRNA level. Further, 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 [16-18], suggesting that the synthesis of the Bβ chain is a rate-limiting peptide for the assembly and secretion of mature fibrinogen.

Down-regulation of mRNA of HepG2 cells using siRNA was performed as a model having heterozygous abnormality, in which mRNA levels are around half those of normal people. When *FGA*, *FGB*, or *FGG* mRNA expression levels were down-regulated by almost 50 %, fibrinogen concentrations in media were 78 %, 49 %, or 57 % of those in the absence of siRNA, respectively; that is to say, when the *FGA* mRNA expression level was decreased by around half, fibrinogen secretion did not decrease by 50 % and remained at approximately 80 %, whereas, under a similar condition, when the *FGB* or *FGG* mRNA expression level was decreased, fibrinogen secretion decreased by approximately 50 %. These results suggest that *FGA* mRNA levels are not limiting fibrinogen production in HepG2 cells. Our experimental data concerning the mRNA levels in the normal liver and down-regulation of mRNA of HepG2 cells suggest that people having heterozygous genetic mutations with a large deletion, nonsense, frameshift, or splicing abnormality in *FGA* (resulting in decreased *FGA* mRNA), show a normal plasma fibrinogen level, but not with *FGB* or *FGG* abnormality.

We showed plasma fibrinogen concentration for probands with afibrinogenemia causing homozygote *FGA*Δ1238bp mutation and compound heterozygotes including *FGA*Δ1238bp mutation, and their heterozygous family members in Table 1 [10-14]. Plasma fibrinogen levels of all heterozygous individuals were almost normal, and 1 of 16 people had <1.5 g/l by either the functional method or immunological determination method. Further, we showed plasma fibrinogen concentrations of probands with afibrinogenemia and their heterozygous family members in Table 2 [11, 19-32]. Genetic mutations in Table 2 included nonsense, frameshift, and splicing abnormality in *FGA*, *FGB*, or *FGG*, all of which lead to premature

termination and NMD [33, 34]. Normal range of plasma fibrinogen was not standardized world-wide, however, it was generally 1.5 - 4.0 or 2.0 - 4.0 g/l, as shown in Table 1 and 2 [10-14, 19-32]. Namely, there is the 2 or 2.7-fold difference between the lower-limit and the upper-limit of the normal range of plasma fibrinogen. Since relatively lower plasma fibrinogen levels in normal range were difficult to evaluate as hypofibrinogenemia, <1.5 g/l of plasma fibrinogen levels was summed up as absolutely low causing of heterozygous genetic abnormalities. For heterozygous individuals with *FGA* mutation, 1 out of 16 people (6.3 %) had <1.5 g/l by either the functional or immunological determination method, whereas in heterozygous individuals with *FGB* or *FGG* mutation, 4 out of 8 people (50.0 %) with *FGB* and 11 out of 12 people (91.7 %) with *FGG* had <1.5 g/l, respectively. Further, we listed plasma fibrinogen levels of hypofibrinogenemia individuals with heterozygous missense mutations, resulting in aberrant fibrinogen assembly and/or secretion (Supplement Table 1). Of interest, the frequency of plasma fibrinogen <1.5 g/l was 85.7 % for *FGA* (6/7), 82.4 % for *FGB* (28/34), and 97.8 % for *FGG* mutation (45/46). Namely, for genetic mutations which did not lead to lower levels of each mRNA than normal, plasma fibrinogen in people with *FGA* mutations was lower than the normal range and its frequency was equivalent to people with *FGB* or *FGG* mutations. Further, in Table 1, Table 2, and Supplement Table 1, frequency of the genetic mutations leading to NMD in afibrinogenemia and/or hypofibrinogenemia patients was 70.0 % for *FGA* (14/20), 19.0 % for *FGB* (4/21), and 14.3 % for *FGG* mutation (4/24), frequency in *FGA* mutation was markedly higher than in others.

Our results indicate that *FGA* mRNA in the normal human liver and HepG2 cells was about 2-fold more than *FGG*, suggest that 1) *FGG* mRNA expression level is limiting but *FGA* mRNA expression level is not in normal liver, 2) when the *FGA* mRNA expression level

in HepG2 cells is decreased by around half, fibrinogen secretion remains approximately 80%, explaining the observation that people with a heterozygous genetic *FGA* abnormality had normal levels of plasma fibrinogen. Although the *FGB* mRNA level in normal human liver was almost the same as *FGA* mRNA (different from the mRNA expression level in HepG2 cells), plasma fibrinogen in people with heterozygous genetic *FGB* mutations was lower than normal. These observations suggest the presence of regulatory mechanisms that reduce the synthesis of Bβ chain polypeptide from *FGB* mRNA [35-37]. In conclusion, we considered that the plasma fibrinogen level might be regulated by the translation level of Bβ and/or γ chain.

Finally, presented data suggest that the normal level of plasma fibrinogen in patients with heterozygous *FGA* mutations, which reduce mRNA, was caused as follows: synthesis of an excess amount of normal Aα chain mRNA (and peptide) from the normal allele compensates for the reduced level of Aα chain mRNA (and peptide) from the mutant allele. In addition, our data also imply that latent individuals with heterozygous genetic *FGA* abnormality might exist much more, because their plasma fibrinogen levels are normal.

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Authorship

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

K. Matsuda, F. Terasawa, M. Sugano, T. Honda and N. Okumura designed the research and reviewed the paper.

Conflict of interest

The authors state that they have no conflicts of interest.

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Table Legends

Table 1 Cases of afibrinogenemic families with *FGA* 1238 bp deletion.

*: EDTA plasma, F: Father, M: Mother, (F): Father-type gene mutation, (M): Mother-type gene mutation, ND: Not determined.

Fibrinogen concentration <2.0 g/l is shown by underlining and <1.5 g/l is shown by double underlining.

Table 2 Cases of afibrinogenemic families with nonsense, frameshift, or splicing abnormality.

F: Father, M: Mother, (F): Father-type gene mutation, (M): Mother-type gene mutation, ND: Not determined.

Fibrinogen concentration <2.0 g/l is shown by underlining and <1.5 g/l is shown by double underlining**.**

Supplement Table 1 Cases of hypofibrinogenemic families with heterozygous missense mutations.

ND: Not determined, a: afibrinogenemia, h: hypofibrinogenemia,

h/d: hypofibrinogenemia/dysfibrinogenemia

Fibrinogen concentration <2.0 g/l is shown by underlining and <1.5 g/l is shown by double underlining.

Figure Legends

Figure 1. Amplification curves of *FGA, FGB,* and *FGG* mRNA.

Expression levels of mRNA were measured by real-time quantitative RT-PCR as described in Materials and Methods. In human normal liver (A), amplification curves showed that expression levels of *FGA* and *FGB* mRNA were approximately equal and 2-fold higher than *FGG* mRNA, respectively. On the other hand, in HepG2 cells (B), the expression level of *FGA* mRNA was approximately 2- or 4-fold higher than *FGG* mRNA or *FGB* mRNA, respectively.

Figure 2. siRNA-mediated down-regulation of fibrinogen mRNA and fibrinogen secretion

The siRNA was transfected into HepG2 cells at various concentrations. Expression levels of mRNA were analyzed by real-time quantitative RT-PCR and concentrations of fibrinogen were determined by ELISA, as described in Materials and Methods. The relative mRNA expression levels (gray columns) and fibrinogen concentration (black columns) are displayed when the non-transfected control value (0 nM siRNA) was set to 100 %. Levels of 3 mRNAs and fibrinogen concentrations decreased in a dose-dependent manner with increasing concentration of siRNA.

Figure 3. Association between 50 % down regulation of mRNA and fibrinogen secretion

The siRNA was transfected into HepG2 cells to obtain mRNA expression decreased by almost 50 %. Expression levels of mRNA were analyzed by real-time quantitative RT-PCR and calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Fibrinogen concentrations were

determined by ELISA, as described in Materials and Methods. The mean values and standard deviations of relative mRNA expression level (gray) and fibrinogen concentration (black) obtained from 3 independent experiments are shown as columns and a bar, respectively. Relative mRNA expression level and fibrinogen concentration were set to 100 % as the non-transfected control value (0 nM siRNA). When *FGA* mRNA was down-regulated to around 50 %, fibrinogen secretion was not decreased, whereas fibrinogen secretion was decreased by 50 % in accordance with the 50 % down-regulation of *FGB* or *FGG* mRNA.

Table 1 Cases of afibrinogenemic families with *FGA* 1238 bp deletion

Case No.	Mutation	Fibrinogen concentration (g/l)				Normal range	Reference No.
		Propositus		Heterozygote			
		Thrombin-time method	Immunological method	Thrombin-time method	Immunological method		
1	c.364+86_510+43del1238 (Δ1238 bp)	< 0.20	0.15*	F 1.91 M 1.91	2.48* 2.92*	1.80 - 3.50	10
2	Δ1238 bp(F) c.54+3A>C(M) (IVS1+3A>C)	< 0.20	0.028	F 3.13 M 2.23	3.11 2.31	1.80 - 3.50	10
3	Δ1238 bp	< 0.1	< 0.1	F 1.9 M 2.0	1.8 2.0	1.5 - 4.0	12
4	Δ1238 bp	< 0.10	< 0.01	F 2.45 M 1.41	3.35 2.60	no description	13
5	Δ1238 bp	< 0.10	< 0.01	F ND M ND	ND ND	no description	14
6	Δ1238 bp(M) c.364+1_4delGTAA(F) (Δ4 bp)	0	0.1	F 2.28 M 2.30 (M)2.18 (F)2.84 (F)3.06 (M)2.25 (M)2.07 (M)3.65	3.4 2.3 3.0 3.1 3.4 2.5 2.2 4.2	2 - 4	11

*: EDTA plasma, F: Father, M: Mother, (F): Father-type gene mutation, (M): Mother-type gene mutation, ND: Not determine
Fibrinogen concentration less than 2.0g/l was showed by underline and less than 1.5g/l showed by double underline.

Table2 Cases of afibrinogenemic families with nonsense, frameshift, or splicing abnormality

Case No.		Mutation	Fibrinogen concentration (g/l) Propositus and homozygote		Heterozygote		Normal range	Reference No.
			Thrombin-time method	Immunological method	Thrombin-time method	Immunological method		
7	***FGA***	p.K144fs	< 0.10	< 0.01	F 2.70 M 2.30	3.20 2.20	no description	19
8		p.Q150X	0 F 0.00	0.0018 0.0021	M 4.15 2.15	2.53 3.24	no description	20
9		p.R168X	Not detected	Not detected	F 1.8 M 2.4	ND ND	1.5 - 4	21
10		c.510+1G>T (IVS4+1G>T)	Not detected	0.27	F 2.5 M 2.3	ND ND	no description	22
11		p.Q203X	0	0.2	F 2.0 M 2.7	2.1 3.0	2 - 4	11
12		p.K238fs	< 0.1	< 0.1	F 1.6 M 2.8	ND ND	2 - 4	23
13		p.T279fs(F) c.510+1G>T(M)	< 0.2	0.023	F 1.8 M 2.8	1.5 2.4	1.5 - 4.0	24
14		p.W334X	0.33	0.50	F 2.07 M 3.84	1.60 3.50	no description	25
15	***FGB***	p.R47X	ND	0.014	F ND M ND	1.51 1.38	1.60 - 4.00	26
16		c.958+13C>T (IVS6+13C>T)	＜0.05	0.0324	F ND M ND	1.61 1.62	1.60 - 4.00	27
17		c.1244+1G>T (IVS7+1G>T)	＜0.05	0.0014	F 1.42 M 1.44	1.90 1.86	1.60 - 4.00	27
18		p.W467X	＜0.50	ND	F 1.50 M 1.70	ND ND	no description	28
19	***FGG***	c.78+5G>A (IVS1+5G>A)	＜0.05	＜0.0002	F 1.26 M 1.32	1.18 1.34	1.60 - 4.00	29
20		c.307+5G>A (IVS3+5G>A)	Not detected	Not detected	F 1.60 M 1.20 1.11	0.70 0.65 0.45	1.60 - 4.00	30
21		c.667-320A>T (IVS6-320A>T)	＜0.05 ＜0.05	0.0001 0.0002	F 0.97 M 1.77 1.21	0.65 1.87 1.69	1.60 - 4.00	31
22		p.E257X	＜0.10	＜0.17	F 0.94 M 0.89 1.44 1.48	1.36 1.34 1.65 1.64	1.50 - 4.00	32

F: Father, M: Mother, (F): Father-type gene mutation, (M): Mother-type gene mutation, ND: Not determined.
Fibrinogen concentration less than 2.0g/l was showed by underline and less than 1.5g/l showed by double underline.

Supplement Table 1 Cases of hypofibrinogenemic families with heterozygous missense mutations

Case No.	Mutation		Phenotype	Fibrinogen concentration (g/l) Propositus		Heterozygote	
				Thrombin-time method	Immunological method	Thrombin-time method	Immunological method
1	***FGA***	p.C55G	h	0.41 0.95	1.05 1.50		
2		p.C55R	h	0.56	1.40		
3		p.C64Y	h	0.93	0.80		
4		p.C64F	h	0.34	0.58		
5		p.M70R	h	1.71	1.61		
6		p.D515N	h	1.33	0.79		
7	***FGB***	p.H97L	h	0.5 - 1.4	1.4		
8		p.M148K	h	1.31	0.95	1.29 0.75 0.84	0.77 0.63 0.58
9		p.C227F	a	Not detected	ND	2.18 3.05	1.80 2.84
10		p.R285H	h	1.4	ND	1.4 1.4 1.4	1.1 1.1 1.0
11		p.R294G	h	1.2	1.0		
12		p.A307S	a	Not detected	ND		
13		p.A307V	h	1.87	1.50		
14		p.D346Y	h	1.1	ND	1.0	ND
15		p.Y356C	h	1.46	1.17		
16		p.N381K	h	1.56	1.48		
17		p.L383R	a	< 0.05	0.0013	1.56 1.14 1.22 1.10 1.24 0.96 1.09 1.14 1.56 1.92	1.90 0.87 1.50 0.96 1.28 1.39 1.44 0.89 1.05 3.03
18		p.G430D	a	< 0.05	0.0159		
19		p.N443S	h	1.3	1.1		
20		p.G444A	h	1.1	1.0	1.1 0.65	0.80 0.60
21		p.G444S	h	0.93 - 1.15	ND		
22		p.G464D	a	0.39	0.35		
23		p.W467G	a	< 0.05 ND	0.00062 0.018	ND ND	1.57 1.86
24	***FGG***	p.C179R	h	0.81	0.87	1.58 0.67	1.41 0.71
25		p.G226V	h	1.5	ND	1.1 0.8 0.9 1.0 0.9 1.4	ND ND ND ND ND ND
26		p.W253C	h	0.7	0.8		
27		p.N256H	h	0.84	0.91		
28		p.N256D	h	1.0	0.7		
29		p.W279G	a	0.2	0.4	1.5	1.5
30		p.A315V	h	0.6	0.6	0.5	0.7
31		p.H333Y	h	0.57	0.86	0.83 1.11 1.07	0.70 0.86 0.78
32		p.S339N	h	0.44	0.58	0.62	1.02
33		p.S339R	h/d	0.5	1.2	0.4	1.5
34		p.D346G	h	0.49	0.90		
35		p.N351I	h/d	0.62	1.16	0.56 1.17	1.20 1.79
36		p.C352Y	h	0.5	0.4	0.7 0.6	0.5 0.6
37		p.W361R	h	1.1	1.1		
38		p.M362I	h	0.4	ND		
39		p.A367D	h/d	0.75	1.37	0.61 0.59	1.30 1.24
40		p.A367V	h	0.70	0.69		
41		p.A367T	h	1.64	1.17		
42		p.N371D	h	0.4	< 0.5		
43		p.N371S	h	1.1	1.0	1.2 0.99	1.4 1.02
44		p.G377S	h	1.53	1.63		
45		p.G378C+ p.T379P	h	0.6	0.8		
46		p.Y380C	h/d	0.38	1.53		
47		p.T397I	h	0.9	0.8		

ND: Not determined, a: afibrinogenemia, h: hypofibrinogenemia, h/d: hypofibrinogenemia/dysfibrinogenemia
Fibrinogen concentration less than 2.0g/l was showed by underline and less than 1.5g/l showed by double under

Figure 1

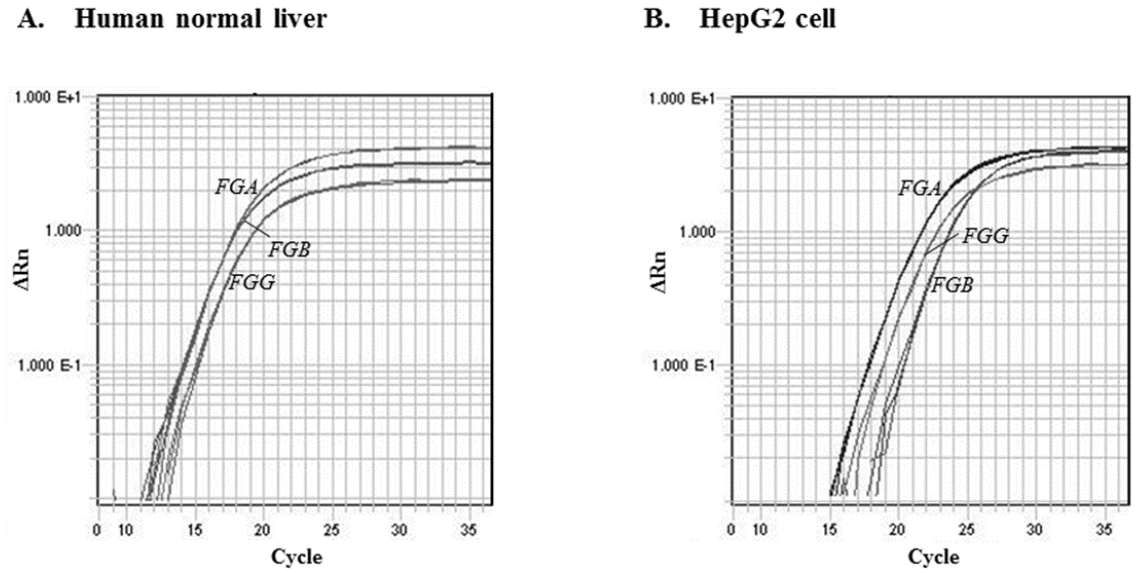


Figure 2

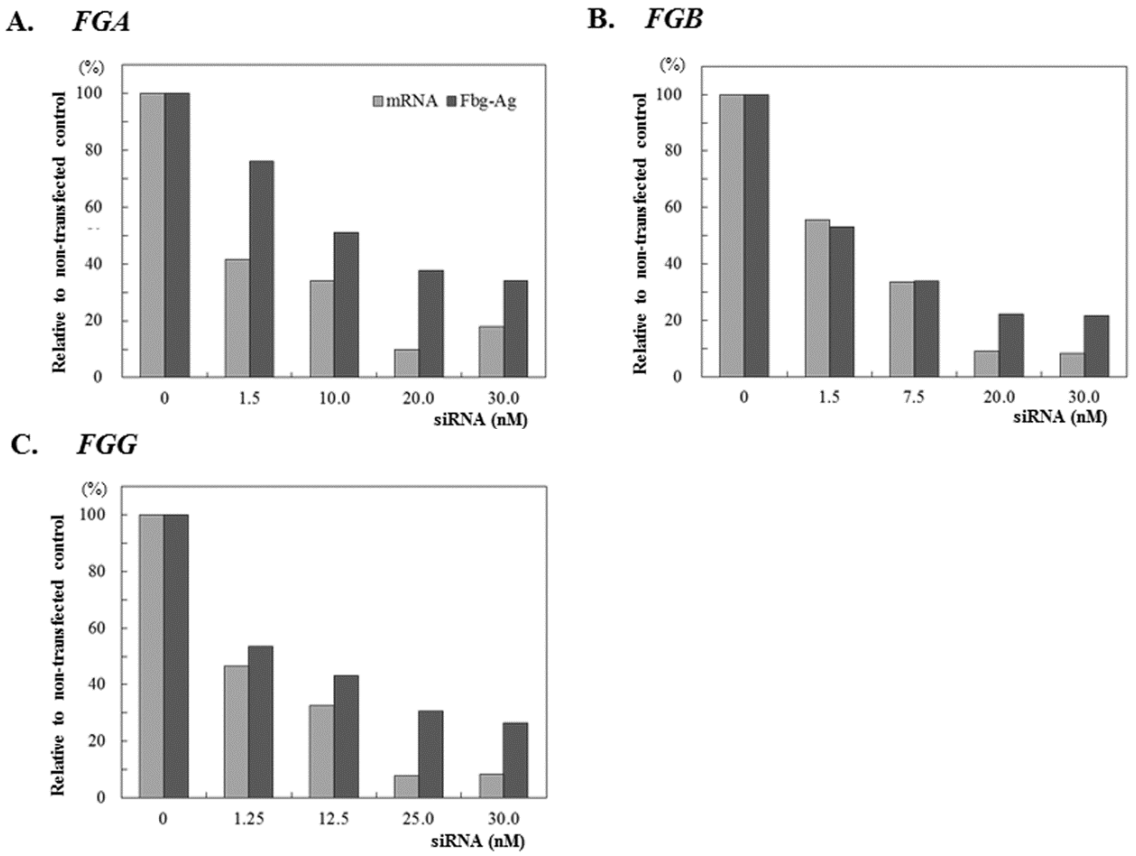
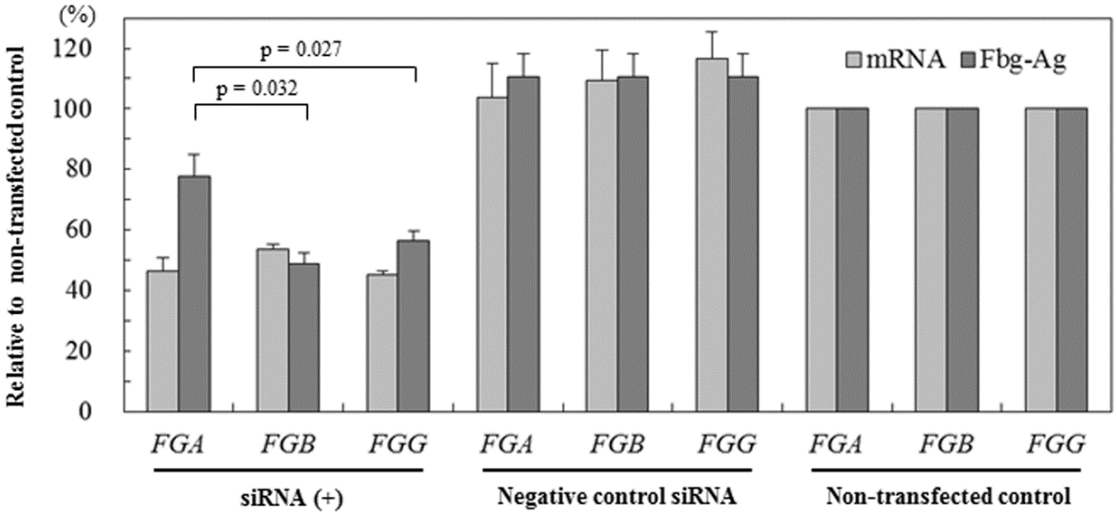


Figure 3


(%)
120
100
80
60
40
20
0
Relative to non-transfected control
p = 0.027
p = 0.032
mRNA
Fbg-Ag
FGA
FGB
FGG
FGA
FGB
FGG
FGA
FGB
FGG
siRNA (+)
Negative control siRNA
Non-transfected control