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

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Abstract

Background: We reported a case of hypofibrinogenemia Matsumoto IX (M IX) caused by a novel compound heterozygous mutation involving an *FGB* IVS6 deletion of 4 nucleotides (Δ4b) (three T, one G; between *FGB* IVS6-10 and -16) and *FGG* IVS3-2A/G, which are both identified for the first time. To examine the transcription of mRNA from the M IX gene, we cloned the wild-type and mutant genes into expression vectors. Methods: The vectors were transfected into CHO cells and transiently produced wild-type, Bβ- or γ-mRNA in the cells. The mRNAs amplified with RT-PCR were analyzed by agarose gel electrophoresis and nucleotide sequencing. Results: The RT-PCR product from *FGB* IVS6Δ4b showed aberrant mRNA that included both introns 6 and 7, and that from *FGG* IVS3-2G showed two aberrant mRNAs, a major one including intron 3 and a minor in which intron 3 was spliced by a cryptic splice site in exon 4. We speculated that the aberrant mRNAs are degraded before translation into proteins, and/or translated variant chains are subjected to quality control and degraded in the cytoplasm. Conclusion: The reduced plasma fibrinogen level of the M IX patient was caused by abnormal RNA splicing of one or both of the *FGB* and *FGG* genes.

1. Introduction

Fibrinogen is a 340 kDa plasma glycoprotein that plays an important role in blood clotting, cellular and matrix interactions, inflammation, wound healing, and neoplasia [1]. Fibrinogen is consisting of a dimeric molecule of 3 polypeptide chains, Aα, Bβ and γ, which are mainly synthesized and assembled into a disulfide-linked hexameric molecule in hepatocytes and secreted into the bloodstream at a concentration of 1.8-3.5 g/L [2]. Aα-, Bβ- and γ-chains are composed of 610, 461 and 411 residues, which are encoded by *FGA*, *FGB* and *FGG*, respectively, that are clustered in a 50 kb region on the long arm of chromosome 4 (4q28-1; *FGG*, -2; *FGA*, -3; *FGB*) [3]. The three genes contain the following numbers of exons: six (included Aα-E isoform) for *FGA*, eight for *FGB* and ten for *FGG*.

Genetic mutations in fibrinogen chain genes have been associated with afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia, as listed in the fibrinogen variant database (http://www.geht.org/databaseang/fibrinogen/), and the molecular bases for the genetic and/or post-translational changes that cause dysfibrinogenemia, hypofibrinogenemia and afibrinogenemia have been described [4, 5]. The prevalence of afibrinogenemia is lower than 1 in 1 million and that of hypofibrinogenemia is more frequent. However the prevalence of hypofibrinogenemia is difficult to establish because of the large number of asymptomatic cases. Mutations of afibrinogenemic patients have been reported about eighty cases, and they are identified in homozygosity or compound heterozygosity [5]. Umbilical cord bleeding is manifested with 85% cases of afibrinogenemia in the neonatal period, whereas, bleeding with a later age-of-onset patient may occur in the skin,

gastrointestinal tract, genitourinary tract, or the central nervous system. Furthermore first-trimester abortion is common in afibrinogenemic women, and paradoxically arterial and venous thromboembolic complications have been reported in afibrinogenemic patients. Hypofibrinogenemic patients are usually asymptomatic, however first-trimester abortion occur in some women [5].

Recently, we identified a novel hypofibrinogenemia associated with compound heterozygous mutations, which were the deletion of 4 nucleotides in *FGB* intron 6 (three T, one G, between *FGB* IVS6-10 and -16; TTTGTTT) and A>G substitution in *FGG* intron 3 (*FGG* IVS3-2 A/G), and designated the condition as Fibrinogen Matsumoto IX (M IX). *FGB* IVS6Δ4b was located near a 3'-intron-exon junction and *FGG* IVS3-2G involved replacement of the consensus splice acceptor site AG to GG [6, 7]. Both of the two mutations have not been reported and are identified for the first time.

Some cases of afibrinogenemia caused by aberrant mRNA have been reported to be associated with large deletions [8-10] or other genetic mutations that affect transcription [11-24]. We assumed that abnormal transcription of mRNA for *FGB* IVS6Δ4b and/or *FGG* IVS3-2G caused hypofibrinogenemia. To determine whether the correct transcription of mRNAs from the M IX mutant genes occurred, we cloned the wild-type and mutant genes of *FGB* and *FGG* into expression vectors, transfected them into mammalian cells, and then analyzed the expressed mRNAs.

2. Materials and methods

This study was approved 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 the biochemical and genetic analyses.

2.1. Data of patient with Matsumoto IX

The patient was a 38-year-old man with type I diabetes mellitus and Hashimoto's disease. He and his family members showed no bleeding or thrombotic episodes, and coagulation test results were as follows: PT 11.7 s (normal range: 10.2-13.4 s), APTT 34.2 s (normal range: 26.2-39.3 s) and functional and antigenic fibrinogen concentrations: 0.83 and 0.82 g/L, respectively (normal range: 1.8-3.5 g/L).

2.2 DNA sequence analysis

Genomic DNA was extracted from whole blood cells using a DNA Extraction WB Kit (Wako Pure Chemical Ltd., Osaka, Japan), according to the manufacturer's instructions. To amplify all exons and exon-intron boundaries in the Aα-, Bβ- and γ-chain genes, 32 PCR primers were designed and the DNA was amplified by PCR as described elsewhere [25]. The PCR products were purified from agarose gels and directly sequenced using a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA). To verify the deletion of the nucleotides or mutation detected by direct sequencing, the PCR products were subcloned into pCR2.1 plasmid vectors using an original TA Cloning Kit (Invitrogen, San Diego, CA, USA) under conditions recommended by the manufacturer, and the extracted subcloned plasmid vectors were sequenced as described above.

2.3 Construction of expression vectors

DNA fragments spanning from intron 5 to the 3' untranslated region (UTR) in exon 8 of the Bβ-chain gene and from intron 2 to exon 5 of the γ-chain gene were amplified from the patient's genomic DNA using the primer couples *FGB*-IVS5-F (5'-GAATGGACAGGG GATTCAGA-3'), *FGB*-UTR-R (5'-CGTCTGCTTGAGAGTTTTAG-3'), *FGG*-IVS2-F (5'-GGAGAGGGGCAGAGGAATAG-3') and *FGG*-Ex5-R (5'-CTTAATGGGTAGCC ACTTTC-3'). PCR-amplified fragments carrying the wild-type of the Bβ- and γ-chain genes were 1548 and 2396 bp, respectively. Purified PCR products of the Bβ- and γ-chain genes were inserted into pTARGET Vector and transfected into JM109 High Efficiency Competent Cells using pTARGET™ Mammalian Expression Vector System (Promega, WI, USA), according to the manufacturer's instructions. Plasmid DNAs were isolated using a miniprep method and sequenced as described above. Four selected clones into which wild- or mutant-type plasmid of the Bβ- or γ-chain gene had been inserted were cultured in a large amount of medium, and the plasmids were isolated using a Qiagen Plasmid Maxi Kit (Qiagen Sci, ML, USA). The base sequences of the plasmids were confirmed and named pT-Bβ-wild-type (wt), pT-Bβ-mutant-type (mt), pT-γ-wt and pT-γ-mt vectors.

2.4 Production of Bβ- and γ-chain mRNAs

The expression vectors pT-Bβ-wt, pT-Bβ-mt, pT-γ-wt and pT-γ-mt were introduced into Chinese hamster ovary (CHO) cells using lipofection. Briefly, 500 μL of antibiotic-free medium containing 8 μg of the vector DNA was mixed with an equal volume of

lipofectamine™ reagent (Invitrogen) (20 μL of lipofectamin diluted with the antibiotic-free medium). After 20 minutes at room temperature, the mixed solution was mixed with approximately 0.5-1 x 10^6 CHO cells in a 6-cm culture dish, according to the manufacturer's protocol. The transfected CHO cells were cultured in 5% CO_2 at 37°C.

2.5 RNA extraction and RT-PCR

The CHO cells were harvested 48 h after transfection. Total cellular RNA was extracted from cells using QIAamp RNA Blood Mini Kit (Qiagen), and contaminated DNA was digested using QIA shredder column and DNase, according to the manufacturer's instructions. Reverse-transcriptase (RT) reactions were carried out in 20 μL of reaction mixture containing 10 μg of the extracted total RNA, 2 μL of RT buffer, 5 μL of 2 mmol/L dNTP mixture, 1 μL of 500 μg/mL oligo dT, 0.2 μL of 0.1 mol/L dithiothreitol and 0.5 μL of 200 U/μL Moloney murine leukaemia virus (M-MLV) with reverse transcriptase transcription at 42°C for 1 h. After the RT reaction, the cDNA was amplified by PCR for 30 cycles using the two pairs of primers that were used in the PCR of the fragment DNA Bβ-chain and γ-chain genes under the following conditions: denaturation at 93°C for 1 min, annealing at 51°C (Bβ-chain) or 56°C (γ-chain) for 1 min, and extension at 72°C for 1 min.

2.6 Analysis of the RT-PCR products by electrophoresis and sequencing

The amplified products were separated by electrophoresis on 2% agarose gels and purified from the gels using Gene Clean II Kit (Funakoshi, Tokyo, Japan). DNA fragments were sequenced as described above using the primers that were used for the PCR of the

patient's genomic Bβ- and γ-chain genes.

3. Results

3.1 DNA sequence analysis of the Matsumoto IX patient

The nucleotide sequence of the Aα-, Bβ- and γ-chain gene-coding region, including exon-intron boundaries, was determined by direct sequence analysis. The sequence showed the deletion of 4 bases (three T, one G) in intron 6 of the Bβ-chain at positions between 6490 and 6496 (TTTGTTT) and a heterozygous A to G transition in intron 3 of the γ-chain at position 761 (all nucleotide positions are numbered taking the starting point of the transcription of the Bβ- or γ-chain gene as 1). The deletion of 4 nucleotides of the Bβ-chain and the nucleotide substitution of the γ-chain were located between IVS6-10 and -16 of the Bβ-chain (*FGB* IVS6Δ4b) and at IVS3-2 of the γ-chain (*FGG* IVS3-2A>G), respectively. It was assumed that these mutations, especially *FGG* IVS3-2A>G and/or *FGB* IVS6Δ4b, may influence splicing of mRNA (Fig. 1-A, B).

3.2 Analysis of fibrinogen Bβ- and γ-chain gene transcripts in CHO cells

To verify whether *FGG* IVS3-2G and/or *FGB* IVS6Δ4b mutations influence transcription of mature mRNAs, mutant Bβ- and γ-chain mRNAs were transiently produced in CHO cells. We constructed 4 expression vectors, pT-Bβ-wt, pT-Bβ-mt, pT-γ-wt and pT-γ-mt, by cloning PCR-amplified genomic DNA fragments of the fibrinogen Bβ- and γ-chains, as described in Materials and methods.

The RT-PCR products from the CHO cells transfected with pT-Bβ-wt showed a major

and a minor band, which were named Bβ-W1 and Bβ-W2, respectively, and those from cells transfected with pT-Bβ-mt showed a single band, named Bβ-M (Fig. 2-A). The RT-PCR products from the CHO cells transfected with pT-γ-wt showed a single band that was named γ-W, and those with pT-γ-mt showed a major and a minor band, which were named γ-M1 and γ-M2, respectively (Fig. 2-B). From the direct sequencing, Bβ-W1 showed a normal mRNA with correct splicing of exon 6 – exon 7 – exon 8; however, for Bβ-W2, exon 7-exon 8 were combined and followed exon 6 for which the initial 18 bases were deleted. For the mRNA of Bβ-M, exon 6 – intron 6 – exon 7 – intron 7 – exon 8 were combined, and so included not only intron 6 but also intron 7. The result indicated that the deletion of 4 nucleotides between IVS6-10 and -16 of the Bβ-chain led to incorrect transcription (Fig. 2-A).

Although the mRNA of γ-W was normal mRNA containing exon 3 – exon 4 – exon 5 (Fig. 2-B), for γ-M1, exon 3 – intron 3 – exon 4 – exon 5 were combined, which constituted abnormal mRNA that included intron 3. For γ-M2, exon 3 – exon 4 – exon 5 were combined, and the product was generated using a cryptic 3’ splice site 9-nt downstream from the beginning of exon 4.

4. Discussion

In this study, we described hypofibrinogenemia M IX associated with 2 novel mutations, one of which is the deletion of 4 nucleotides (three T, one G) situated in *FGB* IVS6-10 to -16 (TTTGTTT) (*FGB* IVS6Δ4b), and the other is an A>G mutation situated at a splicing acceptor site at *FGG* IVS3-2. *FGB* IVS6Δ4b is located a little downstream of *FGB* intron 6

than the acceptor splice site. On the other hand, the *FGG* IVS3-2A>G mutation converts the conserved dinucleotide AG acceptor splice site to GG [6, 7].

To examine whether the cause of the low level of M IX plasma fibrinogen is *FGG* IVS3-2G and/or *FGB* IVS6Δ4b, we analyzed the mRNAs transcribed from the cloned wild-type and mutant *FGB* and *FGG* genes in CHO cells; *FGB* including the region between intron 5 and exon 8 and *FGG* including that between intron 2 and intron 5. Our transient expression system using CHO cells indicated that both *FGB* IVS6Δ4b and *FGG* IVS3-2G mutations induce complete inactivation of the normal 3' splice site. The alternative mRNA from *FGB* IVS6Δ4b was an abnormal product that included both intron 6 and intron 7. In addition the *FGG* IVS3-2G gene generated two mRNA products. The major product (γ-M1) included intron 3 and the minor one (γ-M2) was an aberrant product using a cryptic 3' splice site in exon 4 and lacked 9 bp (ATATGATAGA) from the beginning of exon 4 (coding residues 134-136 of the γ-chain), which resulted in an aberrant γ-chain composed of 408 amino acids. Although two products were seen in the mRNA from Bβ-wt, it was assumed that the minor band (Bβ-W2) was a non-specific PCR product, because the sequence of the forward primer (GAA TGG ACA GGG GAT TCA GA) was similar to the beginning of exon 6 (GAT GGA CAG TGA TTC AGA). We considered that both Bβ-W2 and γ-M2 mRNAs might be produced by artificial transcription that occurred in the CHO cell expression system.

We predicted the amino-acid sequences that would be produced when the aberrant mRNAs of Bβ-M and γ-M1 are translated into proteins. The translational product of Bβ-M was predicted to have substituted aberrant amino acids from the 291st to the 307th residues

and early termination at the 308th. Similarly, the translational product of γ-M1 was predicted to have substituted aberrant amino acids from the 77th to the 99th residues and early termination at the 100th. These aberrant proteins represented truncations of 154 residues from the Bβ-chain and 312 residues from the γ-chain. We could not observe the truncated Bβ- or γ-chain peptide in the patient's plasma by SDS-PAGE and immunoblot analyses using polyclonal fibrinogen antibody and monoclonal Bβ- or γ-chain antibodies under reducing conditions (data not shown); namely, neither truncated Bβ- nor γ-chain could interact with other chains to form a mature hexameric fibrinogen molecule. A previous report demonstrated that the premature termination codons in *FGA* are not associated with decay of the mutant mRNAs [26]. We speculated that the cause of the low level of M IX plasma fibrinogen was that the truncated Bβ and γ-chains are subjected to quality control and degraded by the ubiquitin-proteasome system [27, 28], and/or the aberrant mRNAs are unstable and degraded before translation into proteins, such as via nonsense-mediated mRNA decay in the cytoplasm [29, 30].

Reports on autosomal recessive disorder with a molecular base of congenital afibrinogenemia indicated association with the aberrant splicing caused by inactivation of physiologic splice sites [11-24]. Most abnormal RNA splicing was reported to occur near the donor splice site, with five kinds of mutation in *FGA*, four in *FGB* and three in the *FGG*. On the other hand, mutations at an acceptor splice site were reported in only two cases, one in *FGB* and the other in *FGG* (http://www.geht.org/databaseang/fibrinogen/). Eight kinds of splicing donor site mutations at position IVS+1 were reported (*FGA* four, *FGB* three, *FGG* one), for which an invariable GT dinucleotide of the donor splice site was

changed to TT, AT or CT, which resulted in the generation of aberrant Aα, Bβ or γ-chains through the use of several cryptic splice sites. The most common mutation at position IVS+1 is the Aα IVS4+1G>T mutation, which has been reported in 13 cases. Other cases of splice junction mutation have been reported for *FGA* IVS1+3, *FGB* IVS6+13, *FGG* IVS1+5, *FGG* IVS3+5 and *FGA* IVS3 +1 to +4 deletions (GTAA). Analyses of the *FGA* IVS3 +1 to +4 deletions, which were another *FGA* donor splice site mutation, showed exon 3 skipping in 99% of transcripts, and exon 2 and 3 skipping in 1% of transcripts [23]. On the other hand, mRNAs from *FGB* IVS6Δ4b and *FGG* IVS3-2G, the mutations of both of which were located in acceptor splice sites, showed no splicing out of intron 6 and intron 7 in the Bβ-chain or of intron 3 in the γ-chain, respectively. We consider that the study of these splicing abnormalities reveals important information about mRNA splicing mechanisms.

Fibrinogen M IX is a novel hypofibrinogenemia with compound heterozygous mutation; however, we could not determine whether *FGB* IVS6Δ4b and *FGG* IVS3-2G are present on the same allele or not, because consent for a test to determine this could not be obtained from the patient's family members.

Afibrinogenemias caused by splicing abnormalities have been reported with homozygous mutation, and there is a report that heterozygous splice site mutation of *FGA* does not cause hypofibrinogenemia [14]. We considered that the plasma fibrinogen level might be regulated by the expression level of Bβ- and/or γ-chain, and thus that the reduced plasma fibrinogen level of the M IX patient was caused by abnormal RNA splicing of one or both *FGB* and *FGG* genes.

In conclusion, we reported a case of novel hypofibrinogenemia Matsumoto IX caused by the deletion of 4 *FGB* IVS6 nucleotides and/or *FGG* IVS3-2A>G mutation, both of which are located in an acceptor splice site. Our experimental data indicated that RNA splicing is affected by the mutant alleles for both *FGB* and *FGG*, resulting in the reduced level of plasma fibrinogen in the M IX patient.

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Figure legends

Fig. 1. Nucleotide sequences of fibrinogen Bβ-chain (A) and γ-chain genes (B) in Matsumoto IX (M IX). Genomic DNA: The PCR-amplified Bβ- and γ-chain genes in M IX were sequenced. Subcloning DNA: Wild-type and mutant-type (M IX) genes were cloned. In figure B, two superimposed nucleotides of the wild type T and mutant type C showed exactly the same peak-height at genomic position 761.

Fig. 2. (A) *FGB*, (B) *FGG*. Upper panel: PCR products amplified with forward (F-p) and reverse primers (R-p). Left panel: RT-PCR products separated on a 2% agarose gel. Lane M: DNA size marker. Wt: RT-PCR products amplified from mRNA of transfected wild type. Mt: RT-PCR products amplified from mRNA of transfected mutant type. Right panel: Schematic mRNAs predicted from the wild-type and the mutant-type

genes. (A) W1 was a normal mRNA, and W2 was an aberrant mRNA for which the initial 18 bases of exon 6 from the wild-type *FGB* gene were deleted. M was an aberrant mRNA from the *FGB* IVS6 gene for which 4 nucleotides had been deleted and included intron 6 and intron 7. (B) W was a normal mRNA from the wild-type *FGG* gene. M1 was an abnormal mRNA that included intron 3, and M2 showed that intron 3 was spliced by a cryptic splice site in exon 4 from the *FGG* IVS3+2G gene.