### **Brief report**

# Missense or splicing mutation? The case of a fibrinogen $B\beta$ -chain mutation causing severe hypofibrinogenemia

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The genetic basis of severe hypofibrinogenemia was analyzed in a 57-year-old Italian woman. She turned out to be a compound heterozygote for a novel putative missense mutation (Leu172Gln) and a previously described nonsense mutation (Arg17Stop) in the fibrinogen B $\beta$ -chain gene. The pathogenetic role of Leu172Gln was analyzed by in vitro expression of the mutant recombinant protein in COS-1 cells. These experiments demonstrated that mutant B $\beta$ -Leu172GIn fibrinogen was normally assembled and secreted. Inspection of the nucleotide sequence surrounding the mutation suggested a possible role on pre-messenger RNA (mRNA) splicing. Production of the mutant transcript in HeLa cells confirmed that the mutation activates a cryptic acceptor splice site in exon 4, resulting in a truncated B $\beta$  chain, lacking approximately 70% of the

C-terminal region. This represents the first exonic splicing mutation identified in the fibrinogen genes. These findings strengthen the importance to analyze potentially pathogenetic nucleotide variations at both the protein and the mRNA level. (Blood. 2004; 103:3051-3054)

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### Introduction

Fibrinogen is a plasma glycoprotein mainly synthesized by hepatocytes that circulates as a 340-kDa hexamer consisting of pairs of  $A\alpha$ , B $\beta$ , and  $\gamma$  chains.<sup>1</sup> Its 3-dimensional structure is characterized by 2 lateral nodules (each composed of 2 globular subdomains) joined to a central nodule by coiled-coil triple-helix structures.<sup>2,3</sup> The  $A\alpha$ , B $\beta$ , and  $\gamma$  chains are encoded by 3 different genes (*FGA*, *FGB*, and *FGG*, respectively) clustered on chromosome 4q28.<sup>4</sup> Fibrinogen takes part in hemostasis by forming the fibrin clot and by mediating platelet aggregation. Moreover, it is a class II acute-phase reacting protein.<sup>5</sup>

Congenital fibrinogen defects comprise type I deficiencies (afibrinogenemia and hypofibrinogenemia), with low or not measurable antigen levels, and type II deficiencies (dysfibrinogenemia and hypodysfibrinogenemia), showing normal or altered antigen levels associated with reduced coagulant activity. While dysfibrinogenemias are autosomal dominant diseases, type I deficiencies are inherited as autosomal recessive traits. Patients affected by congenital afibrinogenemia (Mendelian Inheritance in Man, [MIM] #202400) or severe hypofibrinogenemia (MIM \*134820, \*134830, and \*134850) may experience bleeding manifestations varying from mild to catastrophic.<sup>6</sup> The genetic basis of these rare coagulation disorders are invariantly represented by mutations within the fibrinogen genes.<sup>7</sup>

In this study, we analyzed the genetic defects underlying severe hypofibrinogenemia in a proband originally described in 1972.<sup>8</sup>

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The patient was found to be a compound heterozygote for a previously reported nonsense mutation and a novel missense mutation. The latter was demonstrated to exert its pathogenetic effect at the messenger RNA (mRNA) processing level by activating a cryptic acceptor splice site.

### Study design

### Case report and laboratory measurements

The patient is a 57-year-old Italian woman with consistently moderate hemorrhagic symptoms. Plasma fibrinogen concentration was re-evaluated by a functional assay based on fibrin polymerization time<sup>9</sup> and by an enzyme-linked immunosorbent assay (ELISA).<sup>10</sup> Resulting levels (0.2941  $\mu$ M [10 mg/dL] and 0.414681  $\mu$ M [14.1 mg/dL] for functional and immunoreactive fibrinogen, respectively; normal range for both tests, 4.7056-11.764  $\mu$ M [160-400 mg/dL]) were concordant with previous data,<sup>8</sup> confirming the diagnosis of severe hypofibrinogenemia.

## DNA extraction, sequence analysis, in vitro mutagenesis, and cloning of polymerase chain reaction (PCR) products

The patient signed an informed consent according to the Declaration of Helsinki. The study was approved by the institutional review board of the University of Milan. All procedures were performed as described.<sup>11</sup>

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#### **Protein analysis**

Expression of wild-type and mutant fibrinogen was achieved by transiently cotransfecting COS-1 cells with plasmids containing the cDNAs for the 3 fibrinogen chains as described.<sup>12</sup> Pulse-chase experiments, immunoprecipitations, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and evaluation of fibrinogen antigen levels in conditioned media and cell lysates by ELISA were performed as described.<sup>12</sup>

### Analysis of mRNA

A 2125-bp fragment of FGB (introns 2 to 5), PCR-amplified from the proband's genomic DNA using the primer couple FGB-3F 5'-GTCCATGAC-CCAAATCCTTC-3' and FGB-5R 5'-TGTTTCCTGGCATTTTACGG-3', was ligated into the mammalian expression vector pTARGET (Promega, Milan, Italy) as described.<sup>11</sup> Insert orientation and distinction between wild-type (pT-B\beta-In2/In5-wt) and mutant (pT-Bβ-In2/In5-5157T>A) constructs were accomplished by sequencing. Plasmids were independently transfected in HeLa cells (not expressing fibrinogen) by the calciumphosphate technique.<sup>11</sup> One microgram of total RNA extracted 48 hours after transfection (RNAWIZ reagent; Ambion, Austin, TX) was reverse transcribed (Enhanced Avian RT-PCR Kit; Sigma, St Louis, MO), and one tenth of the reverse-transcriptase (RT) reaction was used as template to amplify wild-type or mutant transcripts with the exonic primers FGB-Ex3F 5'-CCTACAGGATGTCAGTTGC-3' and FGB-Ex5R 5'-AACTGTCAG-GTTGAATGAGAT-3'. Quantitative evaluation of transcripts by the fluorescent hot-stop PCR technique was performed as described.11

### **Results and discussion**

DNA sequencing of the fibrinogen cluster revealed the patient to be a compound heterozygote for 2 point mutations in *FGB*. One mutation was a novel T>A transversion in exon 4 at position 5157 (numbering according to GenBank M64983), predicted to cause the Leu172Gln substitution (numbering omits the signal peptide). The other mutation was a C>T transition at position 3282 in exon 2, causing a nonsense mutation at residue 17. This mutation (Arg17Stop) was previously reported in the homozygous state in an Iranian afibrinogenemic patient.<sup>13</sup>

While the causal role of Arg17Stop was self-evident, the pathogenetic role of Leu172Gln required further characterization. First, its absence was checked in 200 haploid genomes from unrelated Italian control individuals. Second, given the unavailability of other family members for segregation analysis, the phase of association of Leu172Gln and Arg17Stop was determined by cloning a 2139-bp region of FGB (nucleotides 3185-5323) from the proband and sequencing 10 recombinant clones. All clones contained either Leu172Gln or Arg17Stop, indicating that the 2 mutations are located on different alleles. Leu172 is located in the second half of the coiled-coil region, whose conserved hydrophobic residues are predicted to play a role in fibrinogen assembly.<sup>14</sup> Although the Leu172Gln substitution is nonconservative (hydrophobic to polar), its impact on fibrinogen assembly and folding is uncertain, since the side chain of Gln172 is comparable with that of the wild-type Leu172 and therefore could fit into the hydrophobic core of the coiled-coil region of fibrinogen.

To confirm the causal role of Leu172Gln, in vitro expression experiments were performed by transiently coexpressing the mutant B $\beta$  protein with the wild-type A $\alpha$  and  $\gamma$  chains in COS-1 cells, which do not express fibrinogen (Figure 1A-B). The 340-kDa fibrinogen band, corresponding to the hexameric molecule, was detectable in both wild-type and mutant samples as well as in control fibrinogen-expressing HepG2 cells, whereas it was absent in mock-transfected cells. These data (confirmed by densitometric quantitation of fibrinogen bands; Figure 1A-B right) demonstrated a normal intracellular assembly and secretion of B $\beta$ -Leu172Gln fibrinogen. Fibrinogen levels in both conditioned media and lysates



Figure 1. In vitro expression of wild-type and Leu172GIn mutant fibrinogens. Immunoprecipitated proteins from conditioned media (A) and cell lysates (B) of COS-1 cells transfected with equimolar mixtures of plasmids driving the expression of the wild-type  $A\alpha$  and  $\gamma$  chains, together with the wild-type or Leu172GIn B $\beta$  chains. Untransfected HepG2 cells and mock-transfected (with the unrelated pUC18 plasmid) COS-1 cells represent the positive and negative controls, respectively. Cells were labeled with [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine for 2 hours and subsequently chased for 0, 30, 60, 120, and 240 minutes. Samples were separated on 4% SDS-PAGE under nonreducing conditions. Labeled proteins were visualized by exposing gels overnight to a storage phosphor screen and analyzed using a Typhoon 9200 phosphor imager and the ImageQuant software (Amersham Pharmacia Biotech, Uppsala, Sweden). The arrowheads indicate the 340-kDa hexameric fibrinogen molecule. A densitometric analysis of the bands corresponding to wild-type ( $\Box$ ) and mutant ( $\Box$ ) immunoprecipitated fibrinogen is shown on the right. Bar graphs are expressed as arbitrary densitometry units (y-axis), as calculated by the ImageQuant software by integrating intensities of all the pixels in the band excluding the background. To compare samples from different panels, each value was normalized using the intensity of the HepG2 fibrinogen band from the same panel. (C) Measurement of fibrinogen antigen levels in conditione media and in cell lysates by means of an ELISA assay, 64 hours after transfection. Bars represent means  $\pm$  SD of 3 independent experiments. Fibrinogen levels are reported as ng/mL (Y-axis).



**Figure 2. Analysis of the effect of the 5157T>A mutation on fibrinogen** B $\beta$  **chain pre-mRNA splicing.** (A) Schematic representation of a region of *FGB* spanning from intron 2 to intron 5. Position and nucleotide sequence of primers used in cloning experiments (*FGB*-3F and *FGB*-5R) and in RT-PCR assays (*FGB*-Ex3F and *FGB*-Ex3F) are reported. Exons and introns are drawn to scale. (B; left) RT-PCR products obtained with the primer couple *FGB*-Ex3F and *FGB*-Ex3F spearated on a 2% agarose gel. Lane M indicates molecular weight marker (pUC8-HaelII); lanes 1-2, RT-PCR products amplified from transfected HeLa cells expressing wild-type or mutant mRNAs, respectively. (Right) Schematic representation of the normal (top) and aberrant (bottom) splicing events. Nucleotide sequences at the exon/intron boundaries are reported. The position of the 5157T>A mutation in exon 4 of *FGB* is indicated by an arrow. Nucleotides surrounding the mutation and matching the acceptor splice site consensus sequence (boxed on the right) are shaded in gray. The region of exon 4 skipped from the aberrant transcript is colored in light gray. The sequence electropherogram confirming the aberrant junction between exon 3 and exon 4 in the RT-PCR product from HeLa cells transfected with the mutant construct is also shown. (C) Semiquantitative analysis of the RT-PCR products as in panel B by means of the fluorescent hot-stop PCR technique. Labeled products were separated on an automated DNA sequencer and analyzed by the GeneScan software. Peak intensities are reported as GeneScan fluorescence units (y-axis). The left and the right panels correspond to RT-PCR products are listed in the table underneath. The percentage values correspond to the peak areas, setting the sum of peak areas of each experiment equal to 100%.

of cells transfected with the mutant plasmid were also evaluated by ELISA and they were even slightly higher than those measured in wild-type samples (Figure 1C), thus convincingly confirming that Leu172Gln does not interfere with fibrinogen synthesis, intracellular processing, and secretion.

Even though Leu172Gln had no apparent effect at the protein level, it still remained a good candidate as causative mutation. Actually, no other putative pathogenetic mutation was found in the sequenced regions of the fibrinogen cluster and the sole presence of Arg17Stop in the proband was not sufficient to account for severe hypofibrinogenemia. In fact, this same mutation was previously reported to be associated with mildly reduced fibrinogen levels in both heterozygous parents of an Iranian afibrinogenemic patient.<sup>13</sup>

A possible effect of Leu172Gln at the RNA level was therefore postulated. A closer inspection of the nucleotide sequence surrounding the mutation revealed that it determines the formation of a sequence (CAGG) that might act as a cryptic acceptor splice site (Figure 2B), causing the skipping of the first 116 nucleotides of exon 4 from the mature transcript.

To test whether Leu172Gln (hereafter referred to as 5157T>A) was itself a splicing mutation, part of the B $\beta$ 

transcript (either wild type or containing 5157T>A) was produced in HeLa cells by transient transfection with 2 minigene constructs (pT-Bβ-In2/In5-5157T>A and pT-Bβ-In2/In5wt; see "Study design" and Figure 2A). RT-PCR on total RNA from cells transfected with pT-Bβ-In2/In5-5157T>A gave rise to a major product (shorter than expected; Figure 2B) and a minor 466-bp product corresponding to the wild-type one, suggesting low levels of residual wild-type splicing. Sequencing revealed that the shorter fragment (350 bp) resulted from the use of a cryptic acceptor splice site generated by the 5157T>A mutation (Figure 2B). This would result in a frameshift and a premature stop at residue 146 of the mutant protein, preceded by a scrambled sequence of 12 residues.

Therefore 5157T>A represents the first exonic splicing mutation identified so far in the fibrinogen genes. A residual wild-type splicing of approximately 10% was calculated by the fluorescent hot-stop PCR technique<sup>11</sup> (Figure 2C). Assuming that this result does mimic the in vivo situation, it might explain the low but measurable level of immunoreactive fibrinogen in the proband's plasma.

These findings strengthen the concept that no safe prediction of phenotypes of mutations is feasible until an accurate functional analysis at the protein and/or RNA level has been performed.

### References

- 1. Doolittle RF. Fibrinogen and fibrin. Annu Rev Biochem. 1984;53:195-229.
- Weisel JW, Stauffacher CV, Bullitt E, Cohen C. A model for fibrinogen: domains and sequence. Science. 1985;230:1388-1391.
- Brown JH, Volkmann N, Jun G, Henschen-Edman AH, Cohen C. The crystal structure of modified bovine fibrinogen. Proc Natl Acad Sci U S A. 2000;97:85-90.
- 4. Marino MW, Fuller GM, Elder FFB. Chromosomal localization of human and rat  $A\alpha$ ,  $B\beta$ , and  $\gamma$  fibrinogen genes by in situ hybridisation. Cytogenet Cell Genet. 1986;42:36-41.
- Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340:448-454.
- Peyvandi F, Duga S, Akhavan S, Mannucci PM. Rare coagulation deficiencies. Haemophilia. 2002;8:308-321.

- Hanss M, Biot F. A database for human fibrinogen variants. Ann N Y Acad Sci. 2001;936:89-90. http://www.geht.org/databaseang/fibrinogen
- Barbui T, Porciello PI, Dini E. Coagulation studies in a case of severe congenital hypofibrinogenemia. Thrombos Diathes Haemorrh. 1972; 28:129-134.
- Clauss A. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol. 1957;17:237-246.
- Cattaneo M, Bettega D, Lombardi R, Lecchi A, Mannucci PM. Sustained correction of the bleeding time in an afibrinogenaemic patient after infusion of fresh frozen plasma. Br J Haematol. 1992; 82:388-390.
- Spena S, Duga S, Asselta R, Malcovati M, Peyvandi F, Tenchini ML. Congenital afibrinogenemia: first identification of splicing mutations in the fibrinogen Bbeta-chain gene causing activa-

tion of cryptic splice sites. Blood. 2002;100: 4478-4484.

- Spena S, Asselta R, Duga S, et al. Congenital afibrinogenemia: intracellular retention of fibrinogen due to a novel W437G mutation in the fibrinogen Bβ-chain gene. Biochim Biophys Acta. 2003; 1639:87-94.
- Asselta R, Spena S, Duga S, et al. Analysis of Iranian patients allowed the identification of the first truncating mutation in the fibrinogen Bβchain gene causing afibrinogenemia. Haematologica. 2002;87:855-859.
- 14. Xu W, Chung DW, Davie EW. The assembly of human fibrinogen: the role of the amino-terminal and coiled-coil regions of the three chains in the formation of the alphagamma and betagamma heterodimers and alphabetagamma half-molecules. J Biol Chem. 1996;271:27948-27953.