Hypofibrinogenemia caused by a novel FGG missense mutation (W253C) in the \( \gamma \) chain globular domain impairing fibrinogen secretion

D Vu, P de Moerloose, A Batorova, J Lazur, L Palumbo, M Neerman-Arbez

**Background:** Inherited disorders of fibrinogen are rare and affect either the quantity (hypofibrinogenemia and afibrinogenemia) or the quality of the circulating fibrinogen (dysfibrinogenemia). Extensive allelic heterogeneity has been found for all three disorders: in congenital afibrinogenemia >30 mutations, the majority in FGA, have been identified in homozygosity or in compound heterozygosity. Several mutations have also been identified in patients with hypofibrinogenemia; many of these are heterozygous carriers of afibrinogenemia null mutations.

**Objective:** To report the case of a patient from Slovakia diagnosed with hypofibrinogenemia characterised by fibrinogen concentrations of around 0.7 g/l.

**Results:** The patient was found to be heterozygous for a novel missense mutation W253C (W227C in the mature protein) in the C-terminal globular domain of the fibrinogen \( \gamma \) chain. Co-expression of the W253C FGG mutant cDNA (fibrinogen Bratislava) in combination with wild-type FGA and FGB cDNAs showed that fibrinogen molecules containing the mutant \( \gamma \) chain can assemble intracellularly but are not secreted into the media, confirming the causative nature of the identified mutation.

**Conclusions:** Current analysis of fibrinogen Bratislava indicates that the domains important for the processes of hexamer assembly and hexamer secretion should not be considered as strictly restricted to one or other fibrinogen chain.

Fibrin is the major protein of the blood clot and is necessary for the mechanical binding of platelets and other plasma proteins, forming a network of fibrin polymers to prevent blood flow from an injured vessel. Fibrin is produced following proteolytic cleavage of fibrinogen by thrombin which allows polymerisation to occur. Fibrinogen is a 340 kDa glycoprotein predominantly synthesised by hepatocytes; it is composed of two sets of three homologous polypeptide chains known as \( \alpha\)-, \( \beta\)-, and \( \gamma \) chains, which assemble to form a hexameric structure (\( \alpha_{2}\beta_{2}\gamma_{2} \)). Each polypeptide is encoded by a distinct gene, FGA, FGB, and FGG, respectively, clustered in a region of 50 kb on chromosome 4q31.

Fibrinogen is normally found in plasma at concentrations of between 1.5 and 3.5 g/l. Inherited disorders of fibrinogen are rare and affect either the quantity (hypofibrinogenemia and afibrinogenemia) or the quality of the circulating fibrinogen (dysfibrinogenemia). More than 400 cases of dysfibrinogenemia have been reported to date, the first dysfibrinogenemia mutation being identified as early as 1968. The majority of dysfibrinogenemias are caused by missense mutations in one of the three fibrinogen genes. Missense mutations at residue R35 (R35H and R35C, or R16H and R16C in the mature protein), which is part of the thrombin cleavage site in the fibrinogen \( \alpha \) chain, are the most common. In contrast, the molecular basis of afibrinogenemia/hypofibrinogenemia has only recently become elucidated.

Congenital afibrinogenemia is characterised by complete deficiency of fibrinogen. In 1999, we identified the first causative mutations for congenital afibrinogenemia: the genetic defect in a non-consanguineous Swiss family was an apparently recurrent deletion of approximately 11 kb of DNA which eliminates the majority of the FGA gene and so leads to a complete absence of functional fibrinogen. Since our identification of the disease locus, numerous causative mutations have been characterised in FGA (the great majority of cases) but also in FGG and FGB, allowing a precise molecular diagnosis for the patients as well as prenatal diagnosis for the families concerned.

Of particular interest are missense mutations leading to complete fibrinogen deficiency: four such missense mutations have been described in FGB: L383R, G430D, G444S, and W467G, numbered from the initiator ATG. These missense mutations all lie in the C terminal portion of the fibrinogen \( \beta \) chain, which is highly conserved among vertebrates. In addition, two nonsense mutations in the same region were recently characterised. The first, W470X, was identified in heterozygosity in an asymptomatic patient. The second nonsense mutation, W467X, three codons upstream, was identified in homozygosity in two Palestinian sisters with afibrinogenemia and results in a fibrinogen \( \beta \) chain which lacks only the last 25 amino acid residues. Expression studies in transfected cells performed for the four missense and the W467X \( \beta \) chain C-terminal mutations both in our laboratory and by others showed that an intact \( \beta \) C-terminal domain is necessary for hexamer secretion into the circulation, although assembly inside the cell appears unimpaired. Interestingly, this property of the fibrinogen \( \beta \) chain seems to differ from the C-terminus of the fibrinogen \( \gamma \) chain, which has been shown to be necessary for intracellular hexamer assembly in experiments investigating serial deletions of the \( \gamma \) C-terminus, but also in studies on hypofibrinogenemia mutants such as fibrinogen Matsumoto IV.

Congenital hypofibrinogenemia is characterised by functional and antigenic fibrinogen levels lower than 1.5 g/l. Although in the past hypofibrinogenemia was considered a separate disorder from afibrinogenemia, with both dominant and recessive modes of inheritance proposed, recent studies (reviewed by Maghazli et al.) have shown that in

**Abbreviations:** ATG, annotated translation start codon; CHO, Chinese hamster ovary
many cases patients are asymptomatic and are in fact heterozygous for null mutations which in homozygosity or compound heterozygosity would cause a fibrinogenaeemia.

In this study, a patient with hypofibrinogenaemia was found to be heterozygous for a novel missense mutation in $FGG$: TGG$\rightarrow$TGT, W253C (W227 in the mature protein) in exon 7. The localisation of this missense mutation close to the previously reported mutations Matsumoto IV$^{13}$ and Hakata$^{14}$ prompted us to carry out co-transfection experiments in COS-7 cells in order to assay its consequence on fibrinogen assembly or secretion and to confirm its causative nature.

**METHODS**

**Clinical description**

The patient, a woman now aged 38, with no family history of bleeding, was diagnosed with hypofibrinogenaemia at the age of 22 years after a bleeding complication of delivery. Her father and mother died at the age of 70 and 63 years, from myocardial infarction and a brain tumour, respectively. The patient suffered from frequent epistaxis during childhood, mild bleeding after tooth extractions, and prolonged wound healing, none however requiring therapeutic intervention. Menarche and subsequent menstrual bleedings were normal. Hypofibrinogenaemia was diagnosed on the basis of low fibrinogen levels (0.7 g/l) after the first spontaneous delivery was complicated by serious bleeding and the development of a large paraecetal haematoma. The patient received three units of red blood cells and a surgical revision was made after an infusion of 3.0 g of fibrinogen concentrate (Fibrinogen, Immuno, Vienna, Austria). At the age of 28 years she had a missed abortion in the first trimester. Mild bleeding occurred again at the age of 31 years after a second spontaneous delivery managed by replacement of 3.0 grams of fibrinogen, which increased the circulating fibrinogen to 1.6 g/l. At the age of 38 years the patient underwent laparoscopic surgery after preoperative administration of 3.0 g fibrinogen, without any bleeding complications.

**Coagulation studies**

Prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), and functional factors II, V, VII, VIII, IX, X, and von Willebrand antigen were measured using reagents, calibrators, and factor deficient plasmas from Dade Behring (Marburg, Germany). Fibrin/fibrinogen degradation products levels were determined with Thrombo-Welcotest (Biotech, Murex, Dartford, UK) and D-dimer with DD-Plus (Dade Behring, Berlin, Germany). Clottable fibrinogen was measured with the Clauss method and fibrinogen antigen with Turbiquant fibrinogen using a Turbitimer (both from Dade Behring). Bleeding time was measured according to Duke, and the euglobulin lysis time (ELT) by standard methods. After obtaining informed consent from the patient, blood was collected in EDTA for DNA analysis.

**Mutation screening**

All exons and intron-exon junctions of the $FGA$, $FGG$, and $FGB$ genes were amplified by polymerase chain reaction (PCR) and sequenced as previously described.$^{5,16}$

**Expression and analysis of the mutations in COS-7 cells**

Construction of wild type and mutant expression vectors, transient co-transfections of COS-7 cells, cell lysate and conditioned media harvesting, and western blot analysis were carried out essentially as previously described,$^{17}$ with some minor modifications. The forward oligonucleotides used for site-directed mutagenesis were: 5'-GCACAACAG AATTTGTCTGGGAATGAGAAG-3' for the Trp253Cys substitution (highlighted in bold); 5'-CCACTATAGAATATAG CCAATTCAACAGACTCAC-3' for the Ile413stop, 5'-GAAGAAAAAC ACTATGATATAATTCCATTACAC-3' for Lys411stop, and 5'-CCGTTGATACCAGTAG AAAAACCATATAG-3' for Lys-406stop with the inserted stop codon in bold. The whole cDNA sequence of all mutants was confirmed by sequencing. Twenty four hours after transfection, cells were washed with phosphate buffered saline (PBS) and incubated for an

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**Figure 1** Western blot analysis of cell extracts and conditioned media of COS-7 cells transfected with fibrinogen cDNAs. Samples of cell lysates and culture medium were subjected to 10% SDS-PAGE under reducing conditions or 7% SDS-PAGE under non-reducing conditions. The blots were incubated with a polyclonal antihuman fibrinogen antibody and cross reaction bands revealed by chemiluminescence. Fg, purified fibrinogen control; $\gamma$, COS cells transfected with the empty vector. The positions of the hexameric complex and the normal $\alpha$, $\beta$, and $\gamma$ chains are indicated. Loading control was carried out with antiaxin antibodies on reduced cell lysates.

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additional 24 hours in media without serum. Conditioned medium with added proteases inhibitors (Complete, Roche) was concentrated using Amicon Ultra-4 5 kDa (Millipore) and cells were scraped in Ripa buffer before addition of reducing or non-reducing 4\textsuperscript{6}Laemmli buffer. Independent experiments were repeated three times. Western blot analysis was undertaken using rabbit antihuman fibrinogen antibodies (DakoCytomation) at a 1:2500 or 1:1500 dilution, or mouse antiactin antibodies (1:2500; Chemicon).

**Figure 2.** Multiple alignment of fibrinogen γ chain and of human fibrinogen αE and Bβ chains in the region surrounding the human W253 residue. Human (P02679-2), mouse (Q8VCM7; from TrEMBL), rat (P02680-2), cow (P12799), chicken (NP_990320; from NCBI), Xenopus (P17634), and lamprey (P04115) fibrinogen γ sequences, as well as human fibrinogen αE (P02671) and Bβ sequences (P02675) were obtained from the Swiss-Prot database, if not otherwise indicated, and aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/). Residue numbering is from the first ATG codon. Identical amino acids are shaded black. The human W253 residue is indicated by the arrow.

**Figure 3.** Crystallographic structure analysis of the γD domain. (A) View of the γC-terminal fragment with the position of W253 (W227 in the mature protein) in the central antiparallel β sheet, with helix highlighted in green, and β strands in orange. (B) W253 (W227) forms two H-bonds (green) with F241 (F215 in the mature protein), while (C) the W253C mutation may create a third H-bond. (D) W253 (W227) is buried and surrounded by polar (yellow) and non-polar (grey) amino acids, within a 4 Å radius. (E) Replacement with a cysteine residue creates a void, possibly leading to local refolding. Figures were prepared using Swiss-PdbViewer and POV-Ray from the PDB file 1FIB.
RESULTS AND DISCUSSION
The patient presented as a classical case of hypofibrino-
genae mia with a proportional decrease in fibrinogen coagu-
lation and antigen levels (0.7 g/l and 0.8 g/l, respectively). She
too had a prolonged thrombin time (31 s, normal range 16–21 s). The aPTT was also slightly prolonged (ratio 1.25, 
normal range 0.8 to 1.2), while PT, other coagulation factor 
levels, and vWF Ag were within the normal range.

Despite moderate fibrinogen deficiency the patient experi-
enced several bleeding episodes as well as a spontaneous 
abortion, which was not explained by examination of the 
placenta or by genetic analysis of the fetus. The bleeding 
episodes were either spontaneous (epistaxis in childhood) 
or provoked (both extractions, cut wounds, postpartum). 
The initial course of the first spontaneous delivery was 
normal; the development of a large pararectal haematoma 
was provoked by the episiotomy probably potentiated by 
hypofibrinogenae mia, with rapid lysis of the weak fibrin clot. 

Haemotherapy with three units of RBC, plasma, and 3.0 g 
fibrinogen prevented the bleeding complications of surgical 
revision of the haematoma. However, mild bleeding occurred 
again following the second spontaneous delivery without 
episiotomy, despite preventive administration of 3.0 g fibrino-
gen which resulted in a circulating fibrinogen level of 
1.6 g/l. In contrast, preoperative infusion of the same dose of 
fibrinogen was sufficient to prevent the bleeding complica-
tion after laparoscopic surgery carried out six years later. This 
supports the suggestion that injury or surgical interventions 
on parenchymous organs with physiologically high fibrino-
lytic potential may require more intensive fibrinogen 
replacement therapy in patients with hypofibrinogenae mia 
and a history of a bleeding tendency.

Sequence analysis of genomic DNA revealed no mutation in 
either $FGA$ or $FGB$; in contrast, a novel missense mutation—
TGG$\rightarrow$TTG, W253C (fibrinogen Bratislava)—was identified in 
$FGG$ exon 7. The localisation of this missense mutation close to 
the previously reported mutations Matsumoto IV$^{11}$ and Hakata$^5$
prompted us to undertake co-transfection experiments in 
COS-7 cells in order to assay its effects on fibrinogen assembly 
and secretion and confirm its causative nature.

The $FGG$ W253C mutant cDNA, obtained by site directed 
mutagenesis of the wild-type $FGG$ cDNA as described in 
Methods, was transiently co-transfected in COS-7 cells with 
wild-type $FGA$ and $FGB$ cDNAs. Three negative controls for 
intracellular hexamer assembly were also produced and 
analysed in the same way. These were $FGG$ I413X, K411X, 
and K406X, corresponding to a deletion of 25, 27, and 32 
residues, respectively, from the $\gamma$ chain C-terminus.$^{12}$ Forty 
eight hours after transfection, individual fibrinogen chains 
(under reducing conditions) or assembled hexamers (under 
non-reducing conditions) in cell extracts and conditioned 
media were detected by western blot analysis with a 
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A Batorova, National Haemophilia Centre, Department of Haematology, University Hospital, Bratislava, Slovakia
J Lazur, Department of Haematology, University Hospital, Kosice, Slovakia

Competing interests: MN-A received a Bayer Haemophilia Project Award.

Correspondence to: Professor Marguerite Neerman-Arbez, Centre Medical Universitaire, 1 rue Michel-Servet, CH-1211 Geneva, Switzerland; Marguerite.Arbez@medecine.unige.ch

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