Congenital hypofibrinogenemia associated with a novel heterozygous nonsense mutation in the globular C-terminal domain of the γ-chain (p.Glu275Stop)

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Key words: hypofibrinogenemia, **γ-**chain, novel mutation, genetic analysis, thrombosis

Dear editors, we wish to report a novel nonsense mutation, p.Glu275Stop, in the fibrinogen **γ-**chain in a patient with congenital hypofibrinogenemia with thrombotic phenotype. To our knowledge, this fibrinogen variant has not been previously reported.

Fibrinogen is a complex glycoprotein comprised of two sets of 3 polypeptide chains (Aα, Bβ, and γ) (Figure 1A), encoded by three genes, i.e. *FGA*, *FGB*, and *FGG*, clustered in a ~50-kb region on chromosome 4q32 [1]. The fibrinogen molecule consists of two outer D regions connected to a central E region by coiled-coil segments. Each region is characterized by different domains with constitutive binding sites that participate in fibrinogen conversion to fibrin, fibrin assembly, crosslinking, and platelet interactions [2]. Congenital hypofibrinogenemia is a quantitative fibrinogen disorder characterised by a proportional decrease of functional and antigenic fibrinogen levels. Hypofibrinogenemic patients are usually asymptomatic according to the fibrinogen level, which however may be associated with low bleeding tendency or, more rarely, with thrombotic complications [3,4]. Hypofibrinogenemia can be considered the phenotypic expression of heterozygous mutations occurring within one of the three fibrinogen genes, mutations that can affect fibrinogen synthesis, assembly, intracellular processing, domain stability, or secretion. Mutations in the fibrinogen cluster, which are present in homozygosity o compound heterozygosity condition, lead instead to congenital afibrinogenemia (Online Mendelian Inheritance in Man #202400). To date, a total of 74 genetic defects have been reported as specifically associated with hypofibrinogenemia: 13 mutations are located in the *FGA* gene, 22 in *FGB*, and 39 in *FGG* [Human Gene Mutation Database, HGMD, http://www.biobase-international.com/product/hgmd; accessed on May 21, 2019]. Mutations accounting for hypofibrinogenemia are relatively frequent in the conserved C-terminal globular domains of the γ and Bβ chains, forming the D region [2,5].

The proband is a Slovak 45-year-old male diagnosed with hypofibrinogenemia upon investigation for recurrent non-provoked deep venous thrombosis of the leg. Subsequently, the patient was set for anticoagulant therapy for secondary thrombosis prevention (warfarin). The patient did not report any significant bleeding episode even during anticoagulation by warfarin.

Coagulation tests revealed normal prothrombin time (PT: 12.3s; Normal range, N: 10.4-12.6s), reptilase time (RT: 20.1s, N: 16.0-22.0s), and activated partial thromboplastin time (aPTT: 22.4s; N: 22.0-32.0s). Thrombin time was slightly prolonged (TT: 21.6 s; N: 12.0-18.0s), functional fibrinogen level, measured using the Clauss method, was 1.3 g/l (N: 1.8-3.5 g/l) and fibrinogen antigen examined by immunoturbidimetric assay was 1.7 g/l. Rotem analyses showed a decreased clot amplitude at 10 minutes in EXTEM (39 mm; N: 43-65 mm) and FIBTEM (4 mm; N: 7-23 mm), as well as a decreased maximum clot firmness (MCF) in FIBTEM (7 mm; N: 9-25 mm) as expected with low functional level of fibrinogen. MCF was borderline in EXTEM (50 mm; N: 50-72 mm). In EXTEM it was observed easy extension of the clotting time (86s; N: 38-79s) (2). The screening tests for thrombophilic mutations (factor V Leiden and prothrombin G20210A) in the patient were negative. However, we observed higher levels of factor VIII (204%; N: 75-120%). With this picture, we suspected a case of congenital hypofibrinogenemia with thrombotic complications. Interestingly, Korte and colleagues describe the total number of congenital fibrinogen disorder patients with thrombosis is 128, but only 16 were specifically suffering from hypofibrinogenemia. Venous thrombosis appears to be the most common thrombotic event [3].

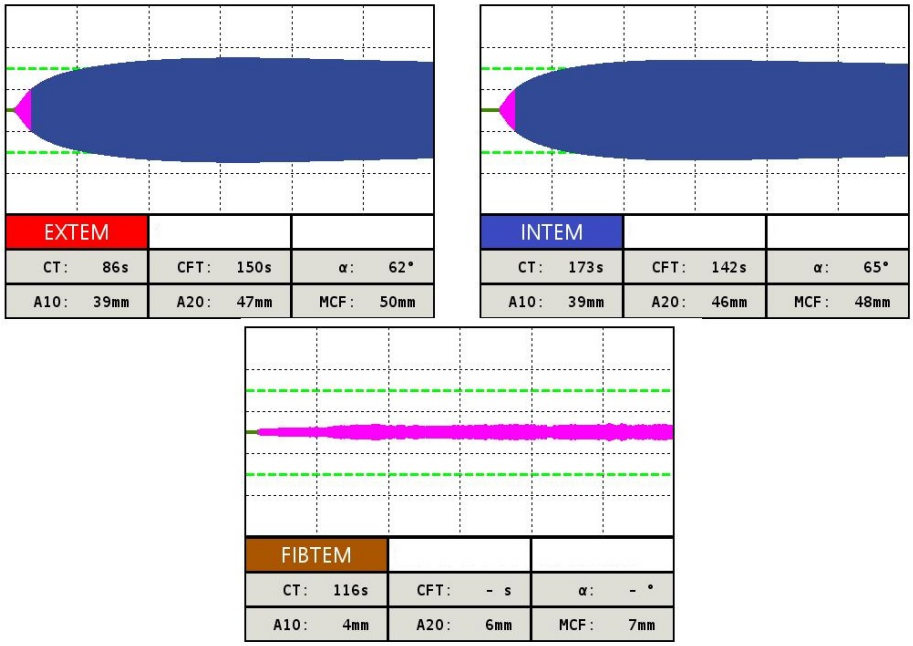

Figure 2. Rotational thromboelastometry results in the patient with hypofibrinogenemia. Panels show the EXTEM (extrinsically activated thromboelastometric test), INTEM (, intrinsically activated thromboelastometric test), and FIBTEM (extrinsically activated thromboelastometric test) data. A10 and A20, clot amplitude 10 or 20 minutes after CT; CFT, clot formation time; CT, clotting time; MCF, maximum clot firmness.

Genetic analysis of all three fibrinogen genes (*FGA*, *FGB*, *FGG*) was performed by Next-Generation Sequencing (NGS). The design of the full-custom AmpliSeq™ Custom DNA Panel for Illumina® was performed using the DesignStudio (https://designstudio.illumina.com; Illumina, San Diego, CA, USA). Probes included all exons, splice junctions, as well as 3´UTR and 5´UTR regions of the three genes. Library preparation was performed according to the manufacturer´s protocol. The amplicon size ranged from 130 to 175 bp, with an average size of 160bp. The high-depth sequencing was performed on the Illumina MiSeq instrument using paired-end reads 2x150nt (we obtained a mean coverage of 250x). Genetic analysis revealed a heterozygous nonsense mutation in exon 7 of the *FGG* gene, c.823G>T (p.Glu275Stop, numbering including the signal peptide). This

mutation was not present in the GnomAD repository (reporting data on >125,000 exomes and >15,000 genomes; https://gnomad.broadinstitute.org/).

Among the 74 single nucleotide substitutions in *FGG* present in HGMD, just 5 are nonsense mutations, and only 3 are located in the globular C-terminal domain of the γ-chain (γC) [7, 8, 9]. Two map in FGG exon 7 and the third in FGG exon 8. Of note, in this same region six missense mutations cluster, and all lead to a specific disorder known as "fibrinogen storage disease". This disorder is characterized by various degrees of hypofibrinogenemia and hepatic inclusions due to impaired release of mutant fibrinogen that accumulates and aggregates in the hepatocellular reticulum [10]. This represents a case of genotype-phenotype correlation in hypofibrinogenemia, and the identification of such type of mutations has a direct clinical implication.

We identified the third nonsense mutation in *FGG* exon 7, p.Glu275Stop. The novel mutation was named "Fibrinogen Martin III" after the town of its discovery. To better understand the molecular anomaly underlying the defect, the effects of the mutation on protein structure were predicted and molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics (http://www.rbvi.ucsf.edu/) (Figure 1).

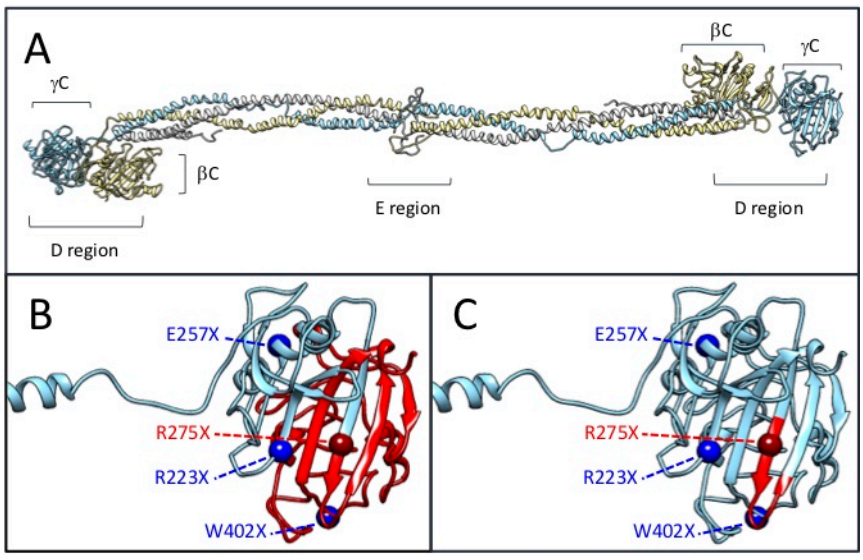

Figure 1 Localization of the p.Glu275Stop mutation within the fibrinogen γ-chain structure. *A*. Schematic representation of the whole structure of fibrinogen based on its crystal structure (6). The three chains (Aα, Bβ, and γ) forming the molecule are shown in *white*, *yellow*, and *light blue*, respectively. The peripheral D regions are composed of the globular C-terminal domains of the βB (βC) and γ (γC) chains. The central E region contains the small fibrinopeptides FpA and FpB that are cleaved by thrombin. *B* and *C*. Inserts showing the positions of the novel nonsense mutation p.Glu275Stop (red sphere) as well as of the other 3 nonsense mutations in γC described in the literature (blue spheres). *B*. The mutation p.Glu275Stop would determine a severely truncated chain, lacking part of the γC (indicated in red). *C*. Alternatively, the RNA mutation is predicted to activate an exonic cryptic donor splice site, with a potential alteration of splicing resulting in a fibrinogen γ-chain molecule bearing an in-frame deletion of 10 amino acids (indicated in red).

Residue 275 is located in the γC and the mutation is predicted to encode a severely truncated chain, lacking part of this conserved domain (Figure 1B). The γC region (residues 143-411) forms a single globular domain and has been proved to play a critical role in fibrinogen assembly and secretion, both in vivo and in cultured cells [8]. A series of γ-chain mutants truncated between residue 379 and the C-terminus demonstrated that residues from 387 to the C-terminus are essentials for fibrinogen intermediate formation and hexamer assembly and thereby secretion from cultured cells [9]. Indeed, γ chains truncated in the coiled-coil domain have been demonstrated to be highly unstable in cell systems, since they are not detected in cell lysates or conditioned media, neither as an individual chain nor as part of a fibrinogen intermediate. At the same time, all described patients who were homozygous for nonsense mutations within the γ-chain present congenital afibrinogenemia [3,11], indicating that the γ chain and the C-terminal domain are critical for fibrinogen secretion from hepatocytes. With these premises, it is highly probable that also fibrinogen molecules bearing the γ-p.Glu275Stop variant could not be competent for secretion. On the other hand, we cannot exclude alternative mechanisms through which some transcripts would skip the stop codon (exon skipping, alternative splicing, codon misreading) in a way to have a secreted fibrinogen with thrombotic potential [3]. To support this hypothesis, we analyzed *FGG* exon 7 sequence

using the Human Splicing Finder web tool (http://www.umd.be/HSF/). Our mutation is predicted to activate an exonic cryptic donor splice site, with a potential alteration of splicing (the predicted newly-created donor site would be AGTgtaact; uppercase letters: predicted exonic nucleotides; lowercase letters: predicted intronic nucleotides; the mutant nucleotide is underlined; score for the donor site: 71.31, over a maximum of 100). In particular, the mutant transcript, if undergoing to the alternative splicing event, is predicted to code for a fibrinogen γ-chain molecule bearing an in-frame deletion of 11 amino acids (Figure 1C).

In conclusion, we report a novel *FGG* mutation leading to hypofibrinogenemia, whose potential deleteriousness was investigated by modeling of the fibrinogen mutant protein, as well as by investigating its possible impact at the transcript level. Further investigations are needed to investigate the thrombotic potential of this novel fibrinogen variant.

Acknowledgements

This work has a general support by departmental chairs. We would like to thank the support of projects Vega 1/0187/17, Grant Comenius University (UK / 102/2017), Martin Center of Biomedicine (BioMed Martin, ITMS 26220220187) and Agency for the Support of Research and Development APVV-16-0020.

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