Pathology (February 2015) **47(2)**, pp. 145–150

HAEMATOLOGY

Identification of a novel splicing mutation in the fibrinogen gamma chain gene leading to dysfibrinogenaemia in a Chinese pedigree

DANDAN HUANG[1,*], HUAYUN CHEN[2,*], XIAOBO HU[2], XUEFENG WANG[3] AND HONGLI WANG[4]

[1]Stem Cell Laboratory, Ningbo No.2 Hospital, Ningbo, Zhejiang Province, [2]Clinical Laboratory Department, No.3 People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, [3]Clinical Laboratory Department, Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, [4]Division of Thrombosis and Hemostasis, Shanghai Institute of Hematology, Shanghai, China; [*]these authors contributed equally to this work and should be considered as co-first authors

Summary

Congenital dysfibrinogenaemia is a rare disease characterised by inherited abnormality in the fibrinogen molecule, leading to functional defects. In this study, we investigated the molecular basis of dysfibrinogenaemia in a Chinese pedigree. Functional fibrinogen of venous blood samples was measured by Clauss method, and the antigens of fibrinogen in plasma were measured by immunoturbidimetry assay. All the exons and exon-intron boundaries of the three fibrinogen genes were analysed by direct sequencing. Fibrinogen electrophoresis, fibrinogen clottability measurement, fibrin polymerisation measurement, and electron microscopy scanning were also used in this study. The proband showed prolonged thrombin time, prolonged reptilase time, reduced fibrinogen activity level and normal plasma fibrinogen antigen. The sequencing results of the proband revealed a novel heterozygous IVS9+1delG mutation of *FGG* gene, which could also be called FGG p.Val434Serfs*29. The same results were also found in her mother, sister and daughter. Both thrombin-induced fibrin polymerisation and reptilase induced fibrin polymerisation were significantly impaired compared to normal control. Fibrinogen clottability measurement showed that only about 38.7% molecules of plasma fibrinogen of the proband were involved in the clot formation. Scanning electron microscopy revealed that the proband's average fibre diameters (159 ± 36 nm; $n = 100$) were found to be significantly thicker than the control's diameter (108 ± 21 nm; $n = 100$) ($p < 0.001$). The fibrin clot of the proband showed irregular structure and highly branched fibres. We conclude that the mutation is responsible for the proband's dysfibrinogenaemia and clinical symptoms.

Key words: Dysfibrinogenaemia, fibrinogen, gene mutation.

Received 11 February, revised 16 September, accepted 25 September 2014

INTRODUCTION

Fibrinogen is a disulfide-bridged protein of 340 kDa comprised of two half-molecules, each having three different polypeptides, designated Aα-, Bβ-, and γ-chains. During coagulation process, thrombin cleaves the amino-termini of the Aα- and Bβ-chains of fibrinogen, releasing fibrinopeptides A and B, and converting fibrinogen to fibrin monomers. The glycoprotein is synthesised in hepatocytes and it has a normal steady plasma concentration of 1.5–3.5 mg/mL with a half-life of approximately 4 days.[1] As the precursor of the clot-forming protein fibrin and as a mediator of platelet aggregation, fibrinogen is an important component of the haemostatic system.[2]

Fibrinogen abnormalities can be classified according to whether there are low or no circulating levels of normal protein (hypofibrinogenaemia or afibrinogenaemia), a mutated species (dysfibrinogenaemia), or a combination (hypodysfibrinogenaemia). Approximately 250 cases of dysfibrinogenaemia have been reported; 55% were asymptomatic (detected by chance), 25% had a tendency to bleeding, and 20% were reported to have a tendency to thrombosis.[3] In this study, we report a case of congenital dysfibrinogenaemia, designated a novel heterozygous mutation, FGG g.IVS9+1delG, p.Val434Serfs*29. This mutation is responsible for impaired fibrinogen clottability and fibrin polymerisation.

MATERIALS AND METHODS

Clinical description

A 33-year-old female patient was found to have prolonged thrombin time, prolonged reptilase time, reduced fibrinogen activity level and normal plasma fibrinogen antigen before a haemorrhoids operation. Three years previously, the patient also experienced haemorrhage during caesarean delivery. The patient had no acute or chronic liver disorder and didn't experience any spontaneous bleeding. The patient, mother, and sister all had a history of menorrhagia. No history of bleeding disorders and consanguineous marriages were described in her family (Fig. 1).

Samples and DNA extraction

Informed consent was acquired from all family members before their participation in the study. This study was approved by the Clinical Research Ethics Committee of No.3 People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

Sodium citrate-anticoagulant peripheral blood samples were withdrawn from all family members and platelet poor plasma (PLT$< 10 \times 10^9$/L) was collected by centrifugation at 2000 g for 10 min. Aliquots of the plasma were immediately analysed or stored at −80 °C until use. Genomic DNA was extracted from leucocytes according to the standard phenol–chloroform protocol.

Coagulation tests

Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and plasma fibrinogen level (Clauss method, with the sensitivity of 0.5 g/L) were tested in all family members using the Sysmex CA7000 Coagulation Analyzer with the accessory reagent kits (Dade Behring, Germany). The plasma Fg concentration was measured by immunoturbidimetry assay on the

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: 10.1097/PAT.0000000000000213

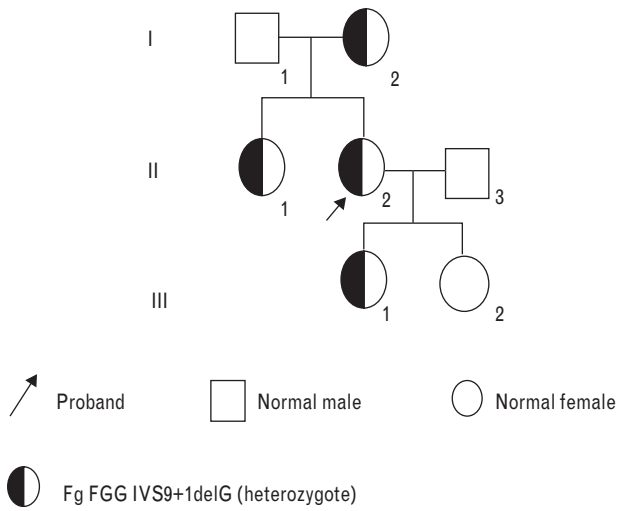

Fig. 1 Pedigree of the family.

Synchron CX7 Super Clinical System (Beckman Coulter, USA) with the reagent kits of Shanghai Sun Biological Products (China) (sensitivity of 0.05 g/L).

Genetic analysis

Primers for PCR were designed according to the published sequences (GenBank accession numbers: M64982, M64983, and M10014), and sequencing primers were in accordance with PCR primers (Table 1). All the exons, exon–intron boundaries and promoter regions of *FGA*, *FGB* and *FGG* were amplified by PCR under standard conditions. Different primer pairs were used to circumvent the risk of false amplification. PCR products were purified from agarose gel with QIAquick Gel Extraction Kit (Qiagen, Germany) and subsequently sequenced on ABI 3700 sequencer (Applied Biosystems, USA). Any mutations identified were confirmed by reverse sequencing. Two alleles from the proband and her mother were separated by cloning into pMD18-T Simple Vector Systems (TaKaRa, China).

Electrophoretic and immunological analysis of fibrinogen

Plasma was diluted with gel loading buffer with 1:200 ratio before boiling for 5 min. Then the sample was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with 8% separating gel and 5% spacer gel (BioRad Minigel System; BioRad Laboratories, USA) under reducing condition, electroblotted onto polyvinylidene difluoride, blocked with casein blocking solution [5% (wt/vol) low-fat milk powder in Tris-buffered saline with Tween-20] and incubated for 2 h at room temperature (RT) with rabbit polyclonal antibody to human fibrinogen (DakoCytomation, Denmark). Antibody staining was visualised with an enhanced chemiluminescence (ECL) Western blotting analysis system (Pierce, USA) using goat anti-rabbit immunoglobulin G (IgG) marked with horseradish peroxidase (HRP) as the second antibody (Cell Signaling Technology, USA). Densitometry analysis was performed using Quantity One software version 4.62 (BioRad).

Fibrinogen clottability measurement

EDTA (0.5 M) was added to platelet poor plasma of the patients and normal control with the aim of attainting the final concentration of 10 mM. Then human thrombin (Rass, China) was added to attain the final concentration of 20 National Institutes of Health (NIH) U/mL and the mixture was incubated for 1 h at 37 °C. The clot was washed with 50 mM pH 7.4 Tris buffer for 5 times, then redissolved in 30% urea solution with the same volume as the plasma. The fibrinogen concentration of the clot was measured with ultraviolet spectrophotometer at 280 nm. The fibrinogen clottability was calculated with the following

$$\text{Fibrinogen clottability } (\%) = \frac{\text{The fibrinogen in the clot}}{\text{The fibrinogen in the plasma (measured with immunoturbidimetry assay)}}$$

Fibrin polymerisation measurement

The plasma fibrinogen level was adjusted to 1 g/L. The rate of fibrin polymerisation was measured after addition of either human thrombin (0.9 NIH U/mL

Table 1 Primers used for PCR and sequencing for *FGA*, *FGB* and *FGG* genes

Primer pair	Forward (5'>3')	Reverse (5'>3')	Amplified/sequencing region
FGA			
1	CTTATAGAAAGCCTTCAGGG	GTGGAAATAAACCACCAGAGAG	Promoter
2	TTTAGAAATATGGATATACCCTC	TAAGAGTGTGTCAGGACATAGAG	Exon1
3	ATCTCTGTGAGAGTGCCAT	TTTCTGGGACCAATCAGGTC	Exon2
4	TGAATCTGAGGATAGATCCTTACTG	TTTATTTAGGATTTTTTGTTGTTTCTG	Exon3
5	CAGCAGCTACTTCAATAACC	GTGCATAACTATCGCCTTCC	Exon4
6	ACCAGGAACTCAATAGACGTAG	AGTTCCAGCTTCCAGCACTG	Exon5-1
7	TATGGAACCGGATCAGAGAC	TGACACCTCTTCAAATGTGCC	Exon5-2
8	ATCTGGAAGTTTTAGGCCAG	TGACAAACTCTCCTAACATAGG	Exon5-3
9	TGATGAAGCTGCCTTCTTCG	AATGACGTGTAACAGAGAG	Exon5-4
10	AGCCGTGCCTATCTTTGTAAAG	TTCATAGGAGGAGACTTGGAG	Exon6-1
11	TCTGGCTAGGCAATGACTAC	AAGACAGAGTGCTCCCATTC	Exon6-2
FGB			
1	CATAGAATAGGGTATGAATT	CCATTTCATAACTATAAGCAAT	Promoter
2	CTGAAGTCATTCCTAGCAGAG	CAATAAGTCAGAGGTTAACAATT	Exon1
3	ATGAGGGTGTTGGAATAGTTAC	TGGGTCAGTGAAACATTAGC	Exon2
4	AATGTCCATGACCCAAATC	ATGCTATTCACTAACCCAGAA	Exon3
5	ATTCTCAGAAAATCAAAATTGTAT	ATGATCTGTTGGGAAAATCC	Exon4
6	ATGTTATTTTAAAGAATTGGTGA	GTATGAGCCACCACACCTG	Exon5
7	ATGGACAGGGGATTCAGATA	TAAAACAGGCTTCCAACAAT	Exon6
8	GGCAGTTTTTAGTTTCCCA	ACAGTAAGTGCCCAGGAAGT	Exon7
9	CTTGACCACCGTAGTTCTGT	GCTTGAGAGTTTTAGAGGAATA	Exon8
FGG			
1	TTTCCAGGGTCATTCATTCC	CCTCTCCAGTTCACACACAA	Promoter
2	GTGCAAAA TCTGGGAACC	AAAGTTACAAGTGCCAGATGA	Exon1,2
3	TAAATATCATCTGGCACTTGTA	ACTTCTATCTCTACTATGCTCAAC	Exon3,4
4	TATTTGTGTTGGGAGTTGAGAC	TTACTTTTCACATCAGCATTCC	Exon5
5	AAGGTTATATTGGGATTAGGTT	TTGCTTATTAGTTATGTGGTCTT	Exon6
6	GCAACCCTAAGAAGTAACCAT	CCAAGAACCAAACAGACTCC	Exon7
7	CCTACGAAAGAGGGAACT	ATGCCAAACTACTGGATA	Exon8
8	ATGCACTTCGTAATAGACAGC	CTTTGTGGGTCAATAGAAGTTA	Exon9
9	TGTCATTTATTTTGTCTTCGTA	ATGGGGTCTTTTGCTCTTA	Exon10