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Hypodysfibrinogenemia: A novel abnormal fibrinogen associated with bleeding and thrombotic complications

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ABSTRACT

Background: Congenital disorders of fibrinogen are rare diseases resulting in the complete absence (afibrinogenemia), reduced concentration (hypofibrinogenemia) or altered function of circulating fibrinogen (dysfibrinogenemia). A combination of two different fibrinogen abnormalities with a significant functional and secretion defect (hypodysfibrinogenemia) reported in Tunisian family members, was investigated in this study.
Methods: The coagulation-related tests, kinetics of fibrin polymerization and lysis and fibrinogen analysis using gel electrophoresis were performed in the family members to characterize fibrinogen abnormalities. All exons including exon-intron boundaries of fibrinogen genes were screened by direct sequencing.
Results: Mutational screening of the fibrinogen genes disclosed novel missense mutations, Bβ Cys197Arg, in exon 4 of the fibrinogen Bβ-chain gene. After the loose of its partner in Bβ-chain, the γCys135 was probably disulfide-bridged to its corresponding Cys residue of another abnormal fibrinogen molecule, forming dimmer with an abnormal electrophoretic profile. Homozygous form carried by the proband found to be directly involved in the bleeding phenotype by affecting fibrin polymerization. In contrast, affected family members bearing the heterozygous mutation showed an impaired fibrin polymerization and fibrinolysis leading to thrombosis.
Conclusion: These results suggest that this mutation could alter the extremely conserved conformations of fibrinogen D domain and *D*-D lateral regions on fibrin assembly.

1. Introduction

Fibrinogen is the focal point of blood clots with a multitude of functions including fibrin clot formation, non-substrate thrombin binding, platelet aggregation and fibrinolysis [1]. It is a plasma 340-kD dimeric glycoprotein secreted by hepatocytes as a complete hexamer composed of two sets of three polypeptides (Aα, Bβ, and γ) linked by 29 inter- and intra-chain disulfide bonds. The six chains are arranged into a trinodular structure which consists of a central E region connected to 2 distal globular D domains by two separate triple helices coiled-coil region. The N-terminal of each chain contributes to constitute the central E domain while the C-terminal of the Bβ and γ chains and a portion of the coiled-coils form the 2 lateral globular D domains. Coiled-coil connectors composed of all three chains link the E and D nodules [2]. Each polypeptide is encoded by a distinct gene, FGA, FGB, and FGG; all three clustered in a region of approximately 50 kb on human chromosome 4q28–31 [3]. Because of its multi-faceted roles in coagulation, quantitative or qualitative modification of this molecule can lead to bleeding or thrombotic phenotypes. Inherited disorders of fibrinogen are rare diseases and affect either the quantity (afibrinogenemia and hypofibrinogenemia; MIM no. 202400), quality (dysfibrinogenemia; MIM no. 616004) or combined defects (hypodysfibrinogenaemia) of circulating fibrinogen. Quantitative fibrinogen deficiencies may result from mutations affecting fibrinogen synthesis, assembly, intracellular processing and protein secretion while qualitative defects are caused

Abbreviations: aPTT, activated partial thromboplastin time; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PT, prothrombin time; SDS, sodium dodecyl sulfate; TT, thrombin time.

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by mutations causing abnormal polymerization, defective cross-linking or defective assembly of the fibrinolytic system [4].

In this study, we report the molecular characterization of a novel homozygous missense mutation within the exon 4 of the fibrinogen beta chain (FGB); p.Cys227Arg identified in Tunisian family with a clinical diagnosis of hypodysfibrinogenemia.

2. Materials and methods

2.1. Patients

All examined subjects signed informed consent after approval of the local Human Ethics Committee and carried out in accordance with the principles of the Declaration of Helsinki.

Three generations of a consanguineous Tunisian family were studied, whose main clinical data and family pedigree are reported in Table 1 and Fig. 1.

2.2. Routine coagulation tests

A final volume of 9 ml blood was collected into plastic tubes containing 1 vol of 3.2% trisodium citrate. Platelet poor plasma (PPP) was separated by centrifugation at 1500*g* for 10 min at 4 °C. Routine coagulation tests (Clauss fibrinogen, Prothrombin time (PT), Thrombin time (TT), D-dimers and activated partial thromboplastin time (aPTT)) were measured with an automated analyzer; STA-R coagulation analyzer (Diagnostica Stago, Asnieres sur Seine, France). Antigenic fibrinogen level was performed in at least triplicate on the same plasma sample using the turbidimetric latex immunoassay (HYPHEN BioMed, West Chester, OH, USA), which is performed on a UV- 2401PC spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Fibrinogen analysis

Fibrinogen was purified by precipitation from plasma with 20% saturated ammonium sulfate. The precipitate was collected by centrifugation and washed twice with 20% saturated ammonium sulfate before being dissolved in water at 37 °C [5]. Purified fibrinogen was analyzed by 7.5% reducing and 4% non-reducing SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and stained with Coomassie Brilliant Blue R-250 according to Laemmli [6]. SDS-PAGE under non-reducing conditions was followed by an immunoblot analysis using rabbit anti-human albumin antibody (Dako, Carpinteria, CA, USA) to investigate albumin binding to fibrinogen. The visualization of cross-reacting species was performed with alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma Chemical Co.).

2.4. Fibrin polymerization curve measurement

Polymerization of fibrin monomer was measured in plasma samples by turbidity changes with time at 350 nm using a Shimadzu UV-260 spectrophotometer. Plasma fibrinogen levels were adjusted to 0.60 g/l, and human thrombin (0.9 NIH U/ml, final concentration) was added at zero time. Samples were run by triplicate in three independent experiments, and the change of turbidity with time was recorded. The lag period and the maximum slope were obtained from the turbidity curves.

2.5. Fibrinolysis

Fibrinolysis was examined in plasma samples after the addition of human thrombin (0.5 NIH U/ml, final concentration), human plasminogen (0.5 U/ml, final concentration), $CaCl_2$ (8 mM, final concentration), and human tPA (0.3 g/ml, final concentration) to 90 μl of diluted plasma samples (diluted to obtain a fibrinogen level of 0.60 g/l) of either the patients or a healthy control. Change in optical density at 350 nm was monitored at one-minute intervals for 45 min.

2.6. DNA analysis

Genomic DNA was extracted from fresh blood cells of the patient and her family members by the salt precipitation method.[7] All exons and the flanking intron regions of the fibrinogen Aα (FGA, GenBank M64982), Bβ (FGB, GenBank M64983), and γ (FGG, GenBank M10014) chain genes were amplified by PCR using a specific primer (metabion international AG, Steinkirchen, Germany). The sequencing was carried out on an ABI Prism 310 DNA sequencer (Applied Biosystems) using BigDye Terminator v.3.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA). Data was analyzed via ABI DNA sequencing analysis software v3.4.1 and mutation detection was performed by Applied Biosystems Variant Reporter™ Software v1.0. After identification of the causative mutation, the related family members were then genotyped for the mutation. To confirm the results, the sequencing was repeated from a separate PCR reaction for each family member.

2.7. Molecular modeling

Molecular modeling was performed with DeepView - Swiss-PdbViewer 4.1 and POV-Ray 3.6 software using the Protein Data Bank file 1fza. The 3D structure file (pdb1fza.ent) was obtained from the EMBL-EBI Macromolecular Structure Database (http://www.ebi.ac.uk/pdbe/entry/pdb/1fza) [2].

3. Results

3.1. Clinical description and family data

The patient was a 45-years-old woman born out of a consanguineous marriage in southern Tunisia (Sfax) (Fig. 1). She presented a severe clinical history of bleeding and discusses the difficulties to achieve appropriate treatment in such severe clinical situation. During her childhood, only moderate bleeding episodes and epilepsy following head trauma were observed. At the age of 18 years old, the patient was hospitalized for severe bleeding after childbirth. When she was 24 years, she had prolonged bleeding after cholecystectomy, two years later at 26 years; she suffered from excessive bleeding during the seventh month of pregnancy.

Table 1
Coagulation test results.

Coagulation tests	III_4	IV_1	IV_2	IV_6	V_1	V_2	V_3	V_4	Normal range
aPTT (s)	42.6	30.4	43.1	40.2	41.4	44.2	41.4	42	26–40 s
Prothrombin time (%)	86	97	62	77	80	72	85	85	70–100
Thrombin time (s)	23.7	16.7	31.2	23.6	22.9	25	23	24.5	16–21 s
Fibrinogen (Clauss) (g/l)	1.68	2.38	0.59	1.09	1.21	1.02	1.46	1.26	2–4 g/l
Fibrinogen (immunoturbidimetric)	1.96	2.41	0.77	1.39	1.53	1.42	1.83	1.60	2–4 g/l
Fibrinogen ratio (activity/antigen)	0.85	0.98	0.76	0.78	0.79	0.71	0.79	0.78	0.85–1.2