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Full Length Article

Congenital afibrinogenemia: Identification and characterization of two novel homozygous fibrinogen Aα and Bβ chain mutations in two Tunisian families

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ABSTRACT

Introduction: Inherited abnormalities of fibrinogen (FG) are rare coagulation disorders divided into two types: quantitative abnormalities (afibrinogenemia and hypofibrinogenemia) or qualitative abnormalities (dysfibrinogenemia and hypo-dysfibrinogenemia) of circulating fibrinogen. In particular, congenital afibrinogenemia is inherited as an autosomal recessive mode and is usually determined by homozygous or compound heterozygous mutations affecting any of the three fibrinogen genes (FGA, FGB and FGG), resulting in the complete absence or extremely reduced amount of fibrinogen. The aim of the present study was to characterize the fibrinogen abnormalities in two Tunisian families.

Methods: Coagulation studies were performed on the patients and family members. All the exons and the flanking intron regions of fibrinogen genes were screened by direct sequencing.

Results: Probands had concomitant bleeding complications with infinitely prolonged standard coagulation assays. Mutational screening of the fibrinogen gene cluster of each proband, disclosed two previously undescribed homozygous point mutations. The first mutation was a major truncation (AαArg252Stop) leads to a severe premature termination codon in the exon 5 of the FGA gene. This mutation defines *in vivo* the importance of the αC flexible segment in the secretion of a stable fibrinogen molecule. The second afibrinogenemic mutation (BβGly295Ala) occurs in the exon 7 of the FGB gene. This missense mutation would probably lead to significant conformational change not allowing the expression of the fibrinogen protein.

Conclusion: Current molecular characterization of these two fibrinogen abnormalities confirms the importance of the first portion of αC-region (αC-connector) as well as the Bβ globular domain in the secretion processes.

1. Introduction

Congenital afibrinogenemia (Mendelian Inheritance in Man, MIM no. 202400) is a very rare autosomal recessive disorder characterized by severe plasma fibrinogen deficiency with an estimated incidence of about one to two per million of the population [1]. Its clinical manifestations vary in severity, ranging from minimal bleeding to catastrophic hemorrhage. All the gene alterations causing afibrinogenemia are located in the three fibrinogen genes (FGA, FGB, and FGG, coding for Aα, Bβ, and γ chain, respectively) clustered in a region of approximately 50 kb on human chromosome 4q28-31 [2]. Fibrinogen is a 340 kDa glycoprotein synthesized predominantly in hepatocytes as a complete hexamer composed of two copies of three homologous polypeptide chains (Aα, Bβ, and γ), interconnected by a complicated series of disulfide bonds [3]. The N-terminus of each chain is disulfide-linked to form the central E region, while the disulfide-linked C-terminal of the Bβ, γ and a portion of Aα chains form two lateral globular D regions. The remaining portion of the C-terminal of the Aα chain (αC region: residues Aα221–610) goes beyond the D region through a flexible segment (αC-connector: residues Aα221–391) to interact with each other and with the central E region forming the αC domain (residues Aα392–610) [4–5]. The crystal structure of fibrinogen molecule demonstrated that Bβ and γ C-terminal domains are two homologous globular entities oriented at approximately 130° to each other. Both of these homologous domains consist of two lateral small subdomains forming a cavity expected for a ligand-binding site and a central domain with a 5-stranded antiparallel β-sheet. A large extended helix interrupted by a snout-like region

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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has been also described in the fibrinogen crystal structure [6]. Molecular analysis of the three fibrinogen genes (Aα, Bβ, and γ) in afibrinogenemic individuals has led to conclude that the most common genetic defects were identified in the Aα chain comparing to Bβ and γ chains [7]. They are mostly described as deletions, insertions, and nonsense mutations causing the insertion of a stop codon.

In this study, two young afibrinogenemic Tunisian patients, each the offspring of a consanguineous marriage, were studied. Sequence analysis of the fibrinogen gene cluster allowed us to identify two previously undescribed homozygous mutations in FGA and FGB gene. A novel Aα chain truncation (AαArg252Stop) was detected in a 9-year-old girl and a novel Bβ missense mutation (BβGly295Ala) in a 3-year-old boy.

2. Materials and methods

2.1. Patients

After receiving all the necessary information about the research, all analyzed individuals signed their consent after approval of the local Human Ethics Committee and carried out in accordance with the principles of the Declaration of Helsinki.

Two unrelated patients from Tunisia, whose main clinical data are summarized in Table 1, were analyzed. The family pedigrees of these patients are reported in Fig. 1.

2.2. Routine coagulation tests

Blood was collected in citrate (1 vol of 3.2% trisodium citrate and 9 volumes of blood). Platelet poor plasma (PPP) was separated by centrifugation at 1500*g* for 10 min at 4 °C and kept frozen until use. Routine coagulation tests (Clauss fibrinogen, prothrombin time (PT), thrombin time (TT), D-dimers, and activated Partial Thromboplastin Time (aPPT)) were performed with citrated plasma on coagulation analyzer; STA-R coagulation analyzer (Diagnostica Stago, Asnieres sur Seine, France). The immunologic fibrinogen level was performed in at least triplicate on the same plasma sample using two different methods: fibrinogen radial immunodiffusion kit (Binding Site Group Ltd, Birmingham, UK) and 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 [8]. 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 [9].

2.4. Mutational screening

Genomic DNA was isolated from peripheral leukocytes using the salt precipitation method [10]. All exons including exon-intron boundaries of fibrinogen genes (FGA, GenBank M64982), (FGB, GenBank M64983), and (FGG, GenBank M10014) were amplified by PCR using GoTaq® G2 DNA Polymerase (Promega, Madison, Wisconsin, USA). Primers (metabion international AG, Steinkirchen, Germany) were designed for the amplification of each exon and the flanking intron regions of fibrinogen genes. Twenty-seven primers were designed for sequence analysis of all exons and exon–intron boundaries. The PCR products were purified and directly sequenced using a BigDye Terminator v. 3.1 cycle sequencing (Applied Biosystems, Foster City, CA, USA), then carried out on an ABI Prism 310 DNA sequencer (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.

2.5. Molecular modeling

Molecular modeling was performed with DeepView - Swiss-PdbViewer 4.1 and POV-Ray 3.6 software using the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) 1FZA.

3. Results

3.1. Proband 1

The first patient was a 9-year-old girl whose parents were first cousins from Northern Tunisia (Zaghouan) (Fig. 1). There is no known case of bleeding disorders in the family. Diagnosis of afibrinogenemia was made at birth after prolonged umbilical cord bleeding and routine coagulation tests that revealed immeasurable functional and immunologic fibrinogen levels in plasma. Since childhood, the patient had a bleeding episode after a tooth extraction. In January 2015, she was referred to the children's Hospital of Tunis for intra-abdominal hemorrhage after a prior appendectomy. To prevent this bleeding, regular infusions of cryoprecipitate were given to maintain fibrinogen levels at >60 mg/dl.

The results of coagulation screening tests of this patient showed: normal platelet count (329,000/mm^3), immeasurable fibrinogen level in both functional and immunologic assays. The TT (control: 16 s) and aPTT (control: 34 s) were infinitely prolonged (Table 1). Both parents, as well as her brother were asymptomatic and had coagulation tests within the normal range.

Reducing and non-reducing SDS-PAGE of purified fibrinogen revealed the complete absence of secreted fibrinogen. In contrast, SDS-PAGE gel analysis showed an essentially normal pattern of hexameric fibrinogen, Aα, Bβ, and γ chains in proband's parents and brother (Fig. 2).

Genetic analysis of the patient's genomic DNA revealed a novel homozygous C to T transition, located in exon 5 of the FGA gene at nucleotide position c.811 (numbering according to GenBank accession number M64982). No other mutation was observed in the patient's FGA gene, or in the FGB and FGG genes. Only a heterozygous 5118G>C substitution responsible for a silent polymorphism (Bβ

Table 1
Coagulation test results.

Coagulation tests	Fibrinogen Zaghouan				Fibrinogen Rdeyef											Normal range
	III-3	III-4	VI-1	VI2	III-3	III-4	VI-1	VI-2	VI-3	VI-4	VI-5	VI-6	V-1	V-2	V-3	
Age (years)	28	35	2	9	67	61	27	24	33	36	26	29	1	3	7	
aPTT (s)	34.7	35.3	36.5	>120	38.8	37.7	42.5	33.5	34.8	38.7	41.4	44.5	41.6	>120	48	26–40 s
Prothrombin time (%)	98	100	100	inc	83	86	69	100	64	89	58	76	86	inc	74	70–100
Thrombin time (s)	16.2	16.6	16.1	>240	23	16.8	26	16.2	22.6	23.2	17	17.6	23.4	>240	21.6	14–21 s
Fibrinogen (Clauss) (g/l)	2.28	2.52	2.56	inc	1.25	2.43	1.19	2.76	1.48	1.32	2.48	2.03	2.15	<0.10	1.42	2–4 g/l
Fibrinogen (immunoreactive) (g/l)	2.40	2.65	2.50	inc	1.30	2.38	1.23	2.95	1.45	1.40	2.62	2.15	2.20	inc	1.52	2–4 g/l

inc denotes fibrinogen incoagulable.