Brief Report

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Prenatal diagnosis for congenital afibrinogenemia caused by a novel nonsense mutation in the FGB gene in a Palestinian family.

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ABSTRACT

Congenital afibrinogenemia is a rare autosomal recessive disorder characterized by the complete absence of detectable fibrinogen. We previously identified the first causative mutations for this disease, these were homozygous deletions of approximately 11 kb of the fibrinogen alpha chain gene (FGA). Subsequent analyses revealed that the great majority of afibrinogenemia alleles are truncating mutations of FGA, although mutations in all three fibrinogen genes, FGG, FGA and FGB have been identified. In this study, we performed the first prenatal diagnosis for afibrinogenemia. The causative mutation in a Palestinian family was a novel nonsense mutation in the FGB gene, W467X. Expression of the W467X mutant FGB cDNA in combination with wild-type FGA and FGG cDNAs showed that fibrinogen molecules containing the mutant beta-chain are not secreted into the media. The fetus was found to be heterozygous for the W467X mutation by direct sequencing and by linkage analysis, a result which was confirmed in the new-born by intermediate fibrinogen levels.

Keywords: prenatal diagnosis, coagulation disorder, fibrinogen, mutation identification and expression
INTRODUCTION

Congenital afibrinogenemia (MIM #202400), characterized by a complete absence of detectable fibrinogen, was originally described in 1920\(^1\) and to date some 150 families with this disorder have been reported\(^2\) with approximately 50% of cases present in consanguineous families\(^3\). Although functional assays of clot formation are infinitely prolonged in affected individuals, the coagulation defect is surprisingly no more severe than in severe hemophilies A and B\(^4,5\). Umbilical cord hemorrhage is often the first sign of the disorder; gum bleeding, epistaxis, menorrhagia, gastro-intestinal bleeding and hemarthrosis occur with varying intensity, and spontaneous intracerebral bleeding and splenic rupture can occur throughout life.

Fibrinogen is synthesized in the hepatocyte from three homologous polypeptide chains, A\(\alpha\), B\(\beta\) and \(\gamma\) which assemble to form the hexameric structure \((A\alpha B\beta\gamma)_2\). The three genes coding for fibrinogen gamma (\(FGG\)), alpha (\(FGA\)) and beta (\(FGB\)) are clustered in a region of approximately 50 kb on chromosome 4q28-q31\(^6\). We previously identified the first causative mutations for this disorder\(^7\) 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 characterized in \(FGA\) (the great majority of cases\(^8,9\)) but also in \(FGG\) and \(FGB\), allowing a precise molecular diagnosis for the patients, as well as prenatal diagnosis for the families concerned. In this study, the first prenatal diagnosis for afibrinogenemia was performed, for a Palestinian family with two affected daughters.
MATERIALS AND METHODS

Description of Family:

Informed consent was obtained from the adults participating in the study. The parents were a healthy consanguineous Palestinian couple with no history of bleeding tendency. Their first conception was a miscarriage during the first trimester. The second and third conceptions produced two daughters who were born at term with birth weights of 2.9 and 3 kg, respectively. The bleeding tendency in the two daughters was noted during mid infancy (between 4-5 months of age) in the form of serious intra-cranial bleeding after non significant trauma. Fibrinogen levels were undetectably low (< 50 mg/dl), establishing the diagnosis of congenital afibrinogenemia. Following diagnosis, treatment consisted of infusions of fresh frozen plasma (FFP) following any significant bleeding, approximately once every 5-6 months. No infusion was required for the last 2 years because of increased parental awareness of accident and trauma prevention. Fibrinogen levels were determined for the parents and were found to be 170 and 150 mg/dl for the mother and the father, respectively.

Mutation screening in family members:

Mutation screening of the FGA, FGG and FGB gene was performed by PCR amplification of all exons and intron-exon junctions followed by sequencing as previously described.8,10

Microsatellite analysis:

The FGA intron 3 tetranucleotide repeat (FGAi3) was analyzed by PCR amplification with oligonucleotides FGA PCR2.1 (5’CCATAGGTTTTGAACTCAG3’) and FGA PCR2.2 (5’CTTCTCAGATCCTCTGACAG3’) followed by denaturing polyacryamide-urea gel electrophoresis according to standard procedures.

Amniocentesis:
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Amniocentesis was performed by the usual procedure at 16 weeks of gestation. Amniotic fluid cells were cultured and after achieving adequate growth, DNA was prepared and analyzed for the mutation present in the affected daughters.

Expression and analysis of the FGB W467X mutation in COS-7 cells:

The three human fibrinogen cDNAs, FGA, FGB and FGG were obtained by RT-PCR on HepG2 cells and cloned into the pcDNA3.1/V5-His TOPO mammalian expression vector (Invitrogen). The W467X mutation was inserted in the FGB construct using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). Transient transfections of COS-7 cells were performed using LipofectAMINE™ Plus reagent (Invitrogen) according to the manufacturer’s instructions. Transfections were performed in 100 mm dishes with 12 µg of the pcDNA3.1/V5-His TOPO vector (negative control) or with equal amounts (4 µg) of all expression vectors. In the case of the heterozygous mutant analysis, 2 µg of each Bβ construct (wild-type and mutant) was used. Three hours after transfection, cells were washed with phosphate-buffered saline (PBS) and incubated for 18 hours in media without serum but with protease inhibitors (Complete Mini, Roche). Conditioned medium was centrifuged to remove cell debris, harvested and concentrated using Ultrafree-4 5K column (Millipore) before adding reducing or non reducing Laemmli buffer. Cells were lysed in 2x concentrated Laemmli buffer. After boiling at 95°C for 5 minutes, samples were analysed by SDS-PAGE (7.5% or 10%). Western blot analysis was performed using rabbit anti-human fibrinogen antibodies (Dako) at a 1:2500 dilution. Immunoreactive bands were revealed with an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech) according to manufacturer’s instructions.
RESULTS AND DISCUSSION

A consanguineous Palestinian couple (first cousins) with two daughters with congenital afibrinogenemia requested a prenatal diagnosis for their ongoing pregnancy (Figure 1A). In the first step, DNA was extracted from fresh EDTA blood samples from parents and affected children for identification of the causative mutation transmitted in this family. Screening of all the coding regions and exon-intron junctions for FGA (\(\alpha\) isoform, exons 1-5) and FGG by PCR and sequencing showed no mutation. Analysis of the FGB gene revealed a previously unreported nonsense mutation in the last exon, exon 8, W467X (TGG>TAG) (numbered from the first ATG codon according to guidelines for human mutation nomenclature\(^{11,12}\)) which was found in heterozygosity in each of the parents, and in homozygosity in each of the two affected daughters (Figure 1B).

Microsatellite analysis of the FGA intron 3 tetranucleotide repeat (FGAi3) was also performed; both parents were found to be heterozygous for the FGAi3 microsatellite marker, while the two affected daughters were homozygous (data not shown).

Mutation analysis was performed on DNA isolated from cultured amniocytes and the fetus was found to be heterozygous for the familial W467X mutation both by sequencing and FGAi3 microsatellite analysis. This result was confirmed in the newborn when the mother gave birth to a healthy baby boy weighing 2.9 kg at birth, and fibrinogen determination revealed a level of 120 mg/dl consistent with heterozygosity for an afibrinogenemia mutation.

The W467X mutation was predicted to lead to the production of a truncated fibrinogen beta chain, with 25 amino acids missing from the C-terminus (Figure 1C). Alternatively, the truncated beta polypeptide might be unstable or the mutation might cause a defect in the FGB mRNA, by aberrant splicing (nonsense-associated alternative splicing) or by affecting the stability of the mRNA through nonsense-mediated mRNA decay\(^{13-15}\).
However, because the W467X mutation lies within the last exon of \textit{FGB}, these mechanisms are not thought to be activated.

Previous studies in COS-1 cells expressing normal fibrinogen alpha and gamma chains in combination with beta-chain deletion mutants had led to the conclusion that the C-terminal portion of the beta-chain, notably residues 238-491 (numbering from the initiator methionine), was not essential for fibrinogen assembly and secretion\textsuperscript{16}. In order to prove the causative nature of the mutation, W467X mutant and wild-type \textit{FGB} cDNAs were transiently co-transfected with wild-type \textit{FGA} and \textit{FGG} cDNAs in COS-7 cells. Eighteen hours after transfection, cells were lysed and the conditioned media harvested. Individual fibrinogen chains and assembled hexamers were detected by Western blot analysis with a polyclonal anti-fibrinogen antibody (Figure 2).

When COS-7 cells are transfected with the three normal fibrinogen cDNAs, all three chains are correctly expressed and assembled inside the cell, and the fibrinogen hexamers are secreted into the media. When cells are transfected with normal \textit{FGA} and \textit{FGG} cDNAs and the W467X \textit{FGB} mutant cDNA, again all three chains are correctly and stably expressed inside the cells. The mutant beta chain is incorporated into fibrinogen hexamer inside the cell (Figure 2B, Cells lane 3) but is not secreted: only incomplete forms containing $\alpha$ and/or $\gamma$ chains, and no fibrinogen hexamer, are detectable in the supernatant (Media A and B, lane 3). These results closely resemble those previously described in COS cells transfected with different combinations of the three normal fibrinogen cDNAs. \textsuperscript{17}

When cells are transfected with equal amounts of wild-type and mutant \textit{FGB} cDNAs (and normal \textit{FGA} and \textit{FGG} cDNAs), imitating heterozygosity for the W467X mutation, both beta chains are clearly distinguished in the Western blot of cell lysates, with the normal chain being more abundant (Figure 2A, Cells lane 2). By contrast, in the cell medium only the wild-type beta chain is found (Fig. 2A, Media lane 2).
The data demonstrate that truncation of the last 25 amino acid residues from the fibrinogen beta chain C-terminus does not inhibit hexamer assembly but eliminates its secretion, as previously reported for two missense mutations in \textit{FGB} exons 7 and 8 identified in afibrinogenemia patients\(^\text{18}\). These results are apparently in contradiction with the experimental observation that fibrinogen beta-chains truncated at amino acid position 238 were able to assemble with alpha and gamma fibrinogen chains and were secreted into the media\(^\text{16}\). However, the assembly and secretion of such a severely truncated polypeptide may not be physiologically relevant.

Interestingly, Homer et al.\(^\text{19}\) reported a very similar mutation (Trp440Stop, amino acids numbered without the signal peptide, or W470X according to our nomenclature) which occurs only 3 codons downstream of the W467X mutation. The W470X mutation “fibrinogen Mount Eden” was identified in heterozygosity in a patient following laboratory investigations prior to a liver biopsy for hepatitis C. The patient had reduced fibrinogen levels (0.7 mg/ml) and a prolonged APTT. Other than mild epistaxis and gum bleeding, the patient was asymptomatic. The authors showed that the truncated fibrinogen beta chain was not found in the patient’s plasma and suggested that removal of the C-terminal 22 residues does not allow incorporation of the mutant chain into mature fibrinogen hexamers. No fibrinogen inclusion bodies were detected in the liver biopsy, indicating that molecules containing the mutant chains do not accumulate in the patient’s hepatocytes. We suggest that the molecular mechanism may be at the level of secretion, as with the W467X mutation we describe. The authors state that the mutation causes hypofibrinogenemia in heterozygosity, however one can consider it an afibrinogenemia mutation since a homozygous individual for this mutation will most certainly have no circulating fibrinogen at all.

In conclusion, the W467X mutation identified in this study, along with the W470X mutation\(^\text{19}\) and three missense mutations\(^\text{18,20}\) identified in the same region in
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afibrinogenemia patients, confirms the necessity of intact C-terminal portions of the fibrinogen beta-chain for the secretion of functional fibrinogen hexamers.

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References

1 Rabe F, Salomon E. Ueber-faserstoffmangel im Blute bei einem Falle von Hämophilie. Arch Int Med. 1920;95:2-14


5 Peyvandi F, Mannucci PM. Rare coagulation disorders. Thromb Haemost. 1999;82:1207-1214


12 den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mut. 2000;15:7-12


Figure Legends:

**Figure 1. Prenatal diagnosis for congenital afibrinogenemia.** A) Family tree. SA: spontaneous abortion. B) Partial sequence of FGB exon 8 demonstrating the FGB W467X (TGG>TAG) mutation. Upper panel, heterozygote; lower panel, homozygote W467X. C) Amino acid sequence of the fibrinogen beta-chain. The Palestinian W467X mutation identified in this study is shown (bold) as well as the W470X “fibrinogen Mount Eden” mutation and three missense mutations L383R, G430D and Y467G. Amino acids encoded by odd-numbered exons (1,3,5,7) are in normal font, those encoded by even-numbered exons (2,4,6,8) are highlighted in grey. Amino acids encoded by codons separated in two consecutive exons are underlined. Amino acids are numbered from the initiator methionine, those included in the signal peptide are in bold.

**Figure 2. 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 A) 10% SDS-PAGE under reducing conditions or B) 7.5% SDS-PAGE under non-reducing conditions. The blots were incubated with a polyclonal anti-human fibrinogen antibody and cross-reacting bands revealed by chemiluminescence, as described in “Material and methods”. Fbg: purified fibrinogen control. –: COS cells transfected with an empty vector. The positions of the hexameric complex and the normal Aα, Bβ and γ chains are indicated; open arrows indicate probable α/γ intermediates (see text and reference 17). An asterisk marks the W467X mutant fibrinogen chain which lacks 25 amino acid residues from the C-terminus. Transfections: lane 1: normal Aα, Bβ, and γ; lane 2: normal Aα, normal Bβ + mutant W467X Bβ, and normal γ; lane 3: normal Aα, mutant W467X Bβ (only), and normal γ.
C  

dehthhet

dehthhom

fibrinogen beta-chain (FGB exons 1-8)

1  MKRMVSWSFH  KLTKMKHLLL  LLLCVFLVKS  QGVNDEEGF  FSARGHRPLD
51  KKREEAPSLR  FAPPPISSGGE  YRARPAKAAA  TQKKVERKAP  DAGGCLHADP
101  DLGVLCPTGC  QLQEALLLQQE  RPIRNSVEDLE  NNNVEAVSQT  SSSSFQYMYL
151  LKDLWQRKQK  QVKNENNEVNN  EYSSELEKHAQ  LIDETVNSN  ITPNLRLIRS
201  ILENLRSTRIQ  KLESDVSAQM  EYCRIPTCTVS  CNIIPVSGKGE  CEEIIRKGGE
251  TSEMYLIPQPD  SSVKPYRYVC  DMNTEENGWCT  VIQNRQDGGSV  DFGKWDHYK
301  QGFGNVATNT  DGNKNCYCLLPQ  EYWLGDKISQ  OITRMPETEL  LIEMEDWKGD
351  KVKAHYGFTP  VQNEANKYQIQ  SVNKYRTGATAG  NEFDSAMQL  GMENRTMTHI
401  NGMFFSTYDR  DNDGWLTSDP  RKQCSKEDCQ  GWNMNRCHAA  NPGRVMWGG
451  QYTWDMAKHP  TDGVVEESMQ  KGSWYSRMQK  SMKRPFPPOQ  W467X

W470X(Trp440stop)
Figure 2

A (reducing)

B (non-reducing)
Prenatal diagnosis for congenital afibrinogenemia caused by a novel nonsense mutation in the *FGB* gene in a Palestinian family

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