Mutational screening of six afibrinogenemic patients: Identification and characterization of four novel molecular defects

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Summary
Congenital afibrinogenemia (CAF) is a rare coagulation disorder characterized by very low or unmeasurable levels of functional and immunoreactive fibrinogen in plasma, associated with a hemorrhagic phenotype of variable severity. It is transmitted as an autosomal recessive trait (prevalence 1:1,000,000) and is invariantly associated with mutations affecting one of the three fibrinogen genes (FGA, FGB, and FGG, coding for α, β, and γ chain, respectively). Fibrinogen is secreted by hepatocytes as a hexamer composed of two copies of each chain; the lack of one chain has been demonstrated to prevent its secretion. Most genetic defects causing afibrinogenemia are point mutations, whereas only three large deletions have been identified so far, all affecting the FGA gene. We here report the molecular characterization of six unrelated afibrinogenemic patients leading to the identification of eight different mutations, four hitherto unknown: a 4.1-Kb large deletion involving exon 1 of FGA (AC107385.g.65682_69828del), two nonsense mutations (p.Trp229X in FGA and p.Trp266X in FGB), and an ins-del mutation (g.1787_1789del3ins12) in FGA. The molecular characterization of CAF-causing genetic defects increases our understanding of the genetic basis of this disease and might be helpful for prenatal screening purposes, as also demonstrated during this study.

Keywords
Fibrinogen, congenital afibrinogenemia, mutational screening, large deletion, point mutations

Introduction
Fibrinogen is a 340-KDa plasma glycoprotein that is converted to insoluble fibrin by thrombin during the last step of the coagulation process (1, 2). The fibrinogen molecule is synthesized and secreted into the bloodstream by hepatic parenchymal cells as a hexamer composed of two identical heterotrimers, each consisting of one α, one β, and one γ chain (1). The three genes (FGA, FGB, and FGG) coding for the fibrinogen chains (α, β, and γ, respectively) are clustered in a 50-Kb region on chromosome 4 (4q32.1) (3).

Inherited fibrinogen disorders are commonly classified on the basis of the plasma concentration of the protein into type I deficiencies (hypofibrinogenemia and afibrinogenemia) with reduced or unmeasurable antigen and functional levels, and type II deficiencies (dysfibrinogenemia and hypo-dysfibrinogenemia) with normal or altered antigen levels associated with reduced coagulant activity. Congenital afibrinogenemia (CAF, OMIM #202400) is a rare autosomal recessive disorder characterized by bleeding manifestations ranging from mild to severe and by the complete absence or extremely low levels of fibrinogen (4). The hemorrhagic diathesis in CAF patients is frequently characterized by umbilical cord bleeding, hemarthrosis, menorrhagia, and post-partum bleeding (4). Moreover, some afibrinogenemic patients show thrombotic complications (5). CAF is a highly heterogeneous genetic disease, 44 causing mutations having been reported within the fibrinogen gene cluster (6): 30 nonsense mutations, five splicing defects, six missense mutations, and three large deletions. Ninety percent of CAF-causing defects gives rise to the afibrinogenemic phenotype by caus...
ing the synthesis of variously C-terminal truncated polypeptides, not competent for secretion.

In this paper we report a study on six unrelated CAF patients and their families. Analysis of the fibrinogen gene cluster revealed eight different mutations, four of them hitherto unknown (one large deletion, two nonsense, one ins-del).

Materials and methods

Patients

Six unrelated CAF patients from Italy (three), Iran (two) and India (one), whose main clinical data are summarized in Table 1, were studied. This study was approved by the Institutional Review Board of the University of Milan. Samples were obtained from the analysed individuals after acquiring an appropriate informed consent.

Proband 1 (P1) is a 48-year-old man from Florence (Italy) who experienced mouth and neck bleeding and was treated with fibrinogen concentrate. Plasma fibrinogen functional and antigen levels were <5 mg/dl and 0.037 mg/dl, respectively. This proband and his family have been previously reported by our group (7); at that time the proband was named F5 and identified as heterozygous for the Gα Gly13ter nonsense mutation.

Proband 2 (P2) is a 78-year-old woman from Palermo (Italy). Her level of plasma functional fibrinogen was below the sensitivity limit of the assay. She suffered from epistaxis, gingival bleeding, cerebral hemorrhage, hematomas, and hemarthrosis. She was prophylactically treated with fibrinogen concentrate before undergoing splenectomy and two dental surgeries. Recently, she was diagnosed with hepatitis C.

Proband 3 (P3) is a 10-year-old boy from Sardinia (Italy) who was clinically diagnosed as afibrinogenemic at birth after a severe sternocleidomastoid muscle hematoma. After that, he suffered from epistaxis and post-traumatic hematomas. The proband’s parents, whose functional fibrinogen levels were 295 mg/dl (mother) and 187 mg/dl (father), are both asymptomatic. The mutational screening of P3 was performed, because the parents recently requested the prenatal diagnosis for their following pregnancy.

Proband 4 (P4) is a man from Mumbai (India) whose fibrinogen plasma functional level was 36 mg/dl.

Proband 5 (P5) is a woman from Shiraz (Iran) who was born from a consanguineous marriage; her plasma fibrinogen functional and antigen levels were <5 mg/dl and <2 mg/dl, respectively.

Proband 6 (P6) is a 12-year-old girl from Shiraz (Iran) whose parents are cousins. Her family has a clinical history of bleeding tendency.

Coagulation tests

Plasma functional and antigen fibrinogen levels were measured by an assay based on fibrin polymerisation time (Laboratoire Stago, Asnières, France) and by an enzyme-linked immunosorbent assay (ELISA) (8), respectively. The sensitivities of the functional and of the immunologic assays were 5 and 0.0005 mg/dl, respectively (normal values for both tests ranged between 160 and 400 mg/dl).

Blood collection and genomic DNA extraction

Venous blood was collected in 1:10 volume of 0.125 M trisodium citrate, pH 7.3. Plasma was obtained by blood centrifugation at 2,000 g for 10 minutes (min), and aliquots were stored at –80°C until use. Genomic DNA was extracted from whole blood using a standard salting-out procedure.

Chorionic villi sampling and genomic DNA extraction

Chorionic villi sampling and genomic DNA extraction were performed according to standard procedures (9, 10).

Sequencing of fibrinogen genes

Sequencing of the FGA, FGB, and FGG coding regions, including exon-intron boundaries and about 500 bp of the promoter regions was performed on PCR fragments amplified from genomic DNA. Oligonucleotides (Invitrogen, Carlsbad, CA, USA) used for sequencing FGA, FGB, and FGG coding regions were designed within introns in order to include exon-intron boundaries. A second set of oligonucleotides was eventually designed within FGA exons to accomplish the complete sequencing of the gene. Primer positions were chosen on the basis of known se-
sequences of the three fibrinogen genes and intergenic regions (GenBank accession numbers M64982, M64983, M10014, U36478, and AC107385). Amplifications were carried out in a Mastercycler Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). PCR conditions and primer sequences can be provided on request. DNA sequencing was performed as previously described (7) using an ABI-3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

### Single nucleotide polymorphisms (SNPs) genotyping

Eleven SNPs in the intergenic region between FGA and FGG were analysed by DNA sequencing (dbSNP reference numbers [11]: rs2066865, rs7659024, rs10034922, rs4696596, rs1984906, rs7659613, rs10050257, rs2070023, rs2070006, rs2070007, and rs10012555). Additional intergenic primers were designed to amplify fragments including these SNPs. PCR conditions and primer sequences can be provided on request.

### Long-range PCR

A 5.5-Kb fragment starting from FGA-FGG intergenic region (position AC107385:g.70675) and ending in FGA exon 2 (position AC107385:g.65175) was amplified using the Expand 20 Kbplus PCR System (Roche Diagnostics, Penzberg, Germany) according to the manufacturer’s instructions. Primers were F1: 5’-CCCTGGAACGTGTGATGAG-3’ and R1: 5’-CCAGTC TTCATCAGAGCAGA-3’. Samples were subjected to a preliminary 2 min denaturation at 92°C, then to 10 cycles of denaturation at 92°C for 10 seconds (sec), annealing at 55°C for 30 sec, and elongation at 68°C for 6 min, followed by 35 additional cycles having a 10-sec increase per cycle of the elongation step. Finally, samples were subjected to 7 min of elongation at 68°C.

### Computer-assisted analyses

DNA sequences were screened for interspersed repeats and low complexity regions with Blat and RepeatMasker software, publicly available at the UCSC genome browser (3).

### Results

#### Mutational screening

Mutational screening was performed in all six investigated probands by sequencing PCR fragments amplified from genomic DNA. Eight different genetic defects were identified (Table 2), four of them not previously reported (Fig. 1).

#### Proband 1

The mutational screening of fibrinogen genes in this proband was previously performed by our group (7). At that time, he was reported as being heterozygous for a transversion identified in exon 2 of the FGA gene (M64982:g.1193G>T) encoding a Gly131ter nonsense mutation; no other genetic defects could be detected within the coding regions of fibrinogen genes. To identify the second mutation contributing to the observed afibrinogenemic phenotype, and that is expected to map within the same gene, we tackled the sequencing of all introns of FGA in this proband. The PCR fragments used to this purpose were obtained with a new set of primers, designed in exonic sequences. No relevant alterations were identified in all analysed introns. Unexpectedly, during the re-sequencing of FGA exon 2 the g.1193G>T mutation was found in the homozygous state. A possible explanation of this unexpected loss of heterozygosity of the FGA gene g.1193 position was the existence of a heterozygous deletion including the entire FGA exon 1 and part of the surrounding areas. We therefore genotyped eleven SNPs in the intergenic region between FGA and FGG to assess the deletion breakpoints (Fig. 2A). The five SNPs (rs10050257, rs2070023, rs2070006, rs2070007, and rs10012555) lying closer to FGA were found in the homozygous state, the first heterozygous SNP being rs7659613. We then PCR amplified a large 5.5-Kb fragment using primers F1 and R1, which were designed in regions known to be heterozygous and thus possibly flanking the hypothesized single-allele deletion (Fig. 2A). Two bands of different length were amplified (Fig. 2B), one corresponding to the wild-
The three genes are in different shades of grey, the horizontal arrows indicate the different transcription orientations and exons (drawn to scale) are depicted as boxes. The position of the four novel mutations is schematically reported on the cluster by the vertical arrows. Amino acids are numbered considering the mature protein. Refer to Table 2 for complete mutation nomenclature and sequence references.

**Proband 4**

This proband was found to be homozygous for the previously reported α-chain gene p.Arg110X mutation (7). The causative transition occurs in FGA exon 4 at position 3075 (M64982: g.3075C>T). The fibrinogen level of 36 mg/dl measured in the patient plasma was unexpectedly high, given the early stop codon and compared to the patient reported by Asselta et al. (7). Unfortunately, patient P4 is from a remote region of India, and we had only one aliquot of plasma for performing fibrinogen quantitation. It was therefore not possible to exclude that the patient had received a replacement therapy before blood withdrawal.

**Proband 5**

DNA sequencing of fibrinogen chain genes in this proband allowed the identification of a novel homozygous G>A transition occurring in exon 5 of FGA (M64982:g.4018G>A). This transition results in a p.Trp229X mutation. The newly identified mutation was found in the heterozygous state in both proband’s parents.

**Proband 6**

In this proband we were able to identify a complex genetic defect occurring in FGA exon 3. The proband is homozygous for a novel deletion/insertion that substitutes three nucleotides with twelve aberrant ones (M64982:g.1787_1789delGTCinsCCC TTGATGTAA). At the protein level this mutation leads to the introduction of three aberrant residues instead of p.Val177, followed by a premature stop codon (p.Val77delinsProLeuMetX). Both parents are heterozygous for this defect.

**Discussion**

In this paper we report the molecular diagnosis of six unrelated afibrinogenemic patients. In four of them we identified four novel CAF-causing genetic defects occurring in the fibrinogen gene cluster: a 4.1-Kb deletion, an ins-del mutation, and a nonsense mutation in FGA, as well as another nonsense mutation in FGB (Fig. 1).

Concerning the novel 4.1-Kb deletion, this defect was detected in the heterozygous state in P1, a CAF proband previously

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**Figure 1: Localisation of the four novel mutations within the fibrinogen gene cluster.** Schematic representation of the fibrinogen gene cluster on chromosome 4 (32.1). The three genes are in different shades of grey, the horizontal arrows indicate the different transcription orientations and exons (drawn to scale) are depicted as boxes. The position of the four novel mutations is schematically reported on the cluster by the vertical arrows. Amino acids are numbered considering the mature protein. Refer to Table 2 for complete mutation nomenclature and sequence references.
reported by our group, whose causative mutations could not be entirely characterized at the moment of the first publication (7). In fact, after having completed sequencing of the coding regions, splice junctions, and about 500 bp of the promoter regions of the three fibrinogen genes, we were only able to identify a single heterozygous g.1193G>T transversion in FGA exon 2 (resulting in the Aα-chain p.Gly13X mutation). This genetic defect was not sufficient to explain the afibrinogenemic phenotype. Therefore, we decided to further investigate this proband for potentially causative non-coding defects by sequencing all FGA introns. This study accidentally led to the identification of a large 5.5-Kb fragment is shown. Position of the g.1193 G>T heterozygous mutation previously identified in Proband 1. Homologous 6 bp-sequences flanking the deletion breakpoints; it’s not possible to determine which one is retained in the deleted allele. MIRb repeat. MIRr repeat. B) Ethidium bromide-stained 1% agarose gel showing the PCR fragments obtained with primers F1 and R1. The smaller deleted allele (~1.4 Kb) is visible in lane P1 together with the wild type one (~5.5 Kb), thus demonstrating the heterozygosity of proband 1 for the ~4.1 Kb deletion. M: molecular weight marker, λ PstI; P1: patient 1 wt: wild-type control. C) Sequence electropherogram of the deleted fragment showing the identification of the breakpoints and the flanking homologous sequence.

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recombination mediated by the short direct repeats, as postulated for the first published CAF mutation, i.e. the 11-kb deletion reported by Neerman-Arbez et al. (16). Moreover, both breakpoints were within a hundred bases from two SINE repeats, which are believed to be potentially involved in the formation of aberrant secondary structures negatively influencing the stability of this genomic tract (17,18). Unlike other diseases, large deletions are rarely reported as responsible for CAF, this deletion being the fourth ever described, and surprisingly they all involve the FGA gene (15, 16, 19). Inspection of the haplotype structure of the genomic region containing the fibrinogen cluster highlighted that recombination rate remains remarkably low throughout the fibrinogen gene cluster, consistently with the overall low number of large deletions reported in this genomic region (6). Notwithstanding that, FGA shows the highest recombination rate within the cluster, possibly explaining the uneven distribution of CAF-causing large deletions (6).

Concerning point mutations, three novel defects causing the synthesis of severely truncated fibrinogen chains were identified in this work. Two of them occur in FGA and one in FGB (Fig. 1). The homozygous transition identified in patient P3 generates a stop codon causing the synthesis of a βB chain lacking 40% of its C-terminus. In particular, βB p.Trp266 lies in the highly structured C-terminal D domain of the βB chain, whose folding is negatively affected by C-terminal truncations altering the shielding of the D-domain hydrophobic core (20). This severe truncation is therefore highly likely to cause the misfolding of the D-domain, which in the end could be responsible for the degradation of the mutant fibrinogen in the ER, as suggested for similar mutations (20). Remarkably, Bβ p.Trp266X is only the third nonsense mutation described in the FGB gene as responsible for CAF (21,22). Curiously, also one of the two previously reported nonsense mutations (22) was identified during prenatal diagnosis in a CAF family, as for the here reported Bβ p.Trp266X.

The PTC substituting Ao p.Trp229 identified in patient P5, determines a severe Aα truncation involving more than 60% of the C-terminus of the protein, while the novel FGA deletion/insertion found in patient P6, encodes the in-frame introduction of three aberrant amino acids followed by a PTC; the resulting Aα chain is only 79-residue-long instead of 625, thus lacking the entire coiled-coil region. Severe fibrinogen chain truncations are the most common cause of CAF and the majority (60%) of them involve the Aα chain. Given that PTCs occurring in the fibrinogen Aα chain do not trigger degradation of the corresponding mRNAs, thus bypassing the nonsense-mediated mRNA decay surveillance (7), these novel mutations are likely to interfere with the assembly and the secretion of fibrinogen molecules rather than with mRNA stability, as demonstrated for similar genetic defects in the same gene (7).

In conclusion, our data provide further hints on the molecular mechanisms generating large deletions in FGA, and confirm the importance of the βB chain C-terminal domain folding for fibrinogen secretion. This study, by reporting four novel genetic defects in the fibrinogen cluster, increases the total number of known CAF mutations by about 10%.

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References
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