

Mutations in the fibrinogen A α gene account for the majority of cases of congenital afibrinogenemia

Marguerite Neerman-Arbez, Philippe de Moerloose, Claire Bridel, Ariane Honsberger, Anni Schönbömer, Colette Rossier, Kathelijne Peerlinck, Ségolène Claeysens, Donna Di Michele, Roseline d'Oiron, Marie Dreyfus, Monique Laubriat-Bianchin, Jacelyne Dieval, Stylianos E. Antonarakis, and Michael A. Morris

Congenital afibrinogenemia is a rare, autosomal, recessive disorder characterized by the complete absence of detectable fibrinogen. We previously identified the first causative mutations in a nonconsanguineous Swiss family; the 4 affected persons have homozygous deletions of approximately 11 kb of the fibrinogen alpha (*FGA*) gene. Haplotype data implied that these deletions occurred on distinct ancestral chromosomes, suggesting that this region may be susceptible to deletion by a common mechanism. We subse-

quently showed that all the deletions were identical to the base pair and probably resulted from a nonhomologous recombination mediated by 7-bp direct repeats. In this study, we have collected data on 13 additional unrelated patients to identify the causative mutations and to determine the prevalence of the 11-kb deletion. A common recurrent mutation, at the donor splice site of *FGA* intron 4 (IVS4 + 1 G > T), accounted for 14 of the 26 (54%) alleles. One patient was heterozygous for the previously identi-

fied deletion. Three more frameshift mutations, 2 nonsense mutations, and a second splice site mutation were also identified. Consequently, 86% of afibrinogenemia alleles analyzed to date have truncating mutations of *FGA*, though mutations in all 3 fibrinogen genes, *FGG*, *FGA*, and *FGB*, might be predicted to cause congenital afibrinogenemia. (Blood. 2000;96:149-152)

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Introduction

Congenital afibrinogenemia (Mendelian Inheritance in Man #202,400) was originally described in 1920.¹ To date some 150 families with this disorder have been reported,² with approximately 50% of instances occurring in consanguineous pedigrees.³ Although functional assays of clot formation are infinitely prolonged in affected persons, the coagulation defect is surprisingly no more severe than in severe hemophilias A and B, varying from severe to moderate.⁴ Umbilical cord hemorrhage is often the first sign of the disorder; gum bleeding, epistaxis, menorrhagia, gastrointestinal bleeding, and hemarthrosis occur with different degrees of intensity, and spontaneous intracerebral bleeding and splenic rupture can occur throughout life. Patients generally respond well to fibrinogen replacement therapy. The genetic defect was assumed to be at the level of fibrinogen synthesis because the half-life of infused fibrinogen is essentially normal.² The fibrinogen locus is comprised of 3 genes coding for fibrinogen gamma (*FGG*), fibrinogen alpha (*FGA*), and fibrinogen beta (*FGB*), clustered in a region of approximately 50 kb on chromosome 4q28-q31.⁵ We previously studied this region in a Swiss family with 2 pairs of affected brothers by using microsatellite analysis, polymerase chain reaction (PCR) amplification, and Southern blotting, identifying the first causative mutations for the disorder.⁶ We found that the genetic defect in this family was an apparently recurrent deletion of approximately 11 kb of DNA that eliminates most of the *FGA* gene,

leading to an absence of functional fibrinogen. We subsequently identified the molecular mechanism involved in the generation of the deletions responsible for congenital afibrinogenemia by cloning and sequencing the deletion junctions for all mutated chromosomes. The deletions were all identical to the base pair and probably resulted from nonhomologous (illegitimate) recombination, mediated by a direct 7-bp repeat, AACTTTT, and perhaps also by indirect repeats in the breakpoint region.⁷

In this study, we performed mutation analysis in the *FGA* gene in 13 additional unrelated patients with congenital afibrinogenemia from Puerto Rico, France, Pakistan, Belgium, and the United States. One patient, from the United States, was a heterozygous carrier of the originally described 11 kb deletion, as revealed by Southern blot analysis. In addition, we identified the most common mutation, in the invariant GT dinucleotide of the donor splice site of *FGA* intron 4 (IVS4 + 1 G > T), which accounted for 14 of the 26 (54%) afibrinogenemia alleles analyzed in this study. Five new mutations leading to premature protein truncation were also identified: 34insC (exon 1), 3121del AA (exon 4), and 4329del C, G316X, and W334X (all exon 5). Finally, a potential IVS1 + 3 donor splice site mutation was identified. All the mutations in patients of Caucasian origin were identified; in contrast, no mutations were found in *FGA* in the 2 non-Caucasian patients studied.

From the Division of Medical Genetics, University Medical School and University Hospital, and the Division of Angiology and Hemostasis, University Hospital, Geneva, Switzerland; the Center for Molecular and Vascular Biology, Leuven, Belgium; the Hemophilia Center, Hôpital Purpan, Toulouse, France; the Department of Pediatrics, Weill Medical College of Cornell Hospital, New York, New York; the Hemophilia Center and the Hematology Laboratory, Hôpital Bicêtre, AP-HP Le Kremlin-Bicêtre; the Centre de Transfusion, Annecy; and the Hematology Laboratory, CHR Amiens, France.

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Reprints: Marguerite Neerman-Arbez, Centre Médical Universitaire, 1 rue Michel Servet, CH-1211 Geneva, Switzerland; e-mail: marguerite.arbez@medecine.unige.ch.

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Patients, materials, and methods

Patients

Clottable fibrinogen levels were measured by the method of Claus.⁸ Fibrinogen concentrations were determined either by radial immunodiffusion, by rocket immunoelectrophoresis, or by nephelometry.^{9,10} Prothrombin time, activated partial thromboplastin time, thrombin time, and clotting factors were measured locally using standard methods. Blood was collected from 13 unrelated patients with congenital afibrinogenemia, as diagnosed by functional or antigenic assays. There was known consanguinity for 3 of the families (Table 1). Informed consent was obtained from all patients participating in the study.

Mutation screening

Genomic DNA was purified from blood leukocytes according to standard protocols. The 5 exons encoding the fibrinogen α isoform were amplified from genomic DNA by PCR, under standard conditions. The alternatively spliced exon 6 was excluded from this study because it persists only in the mRNA encoding the longer α_F isoform, which represents just 1% of adult fibrinogen.¹¹ Exons 2 and 3 were coamplified in a single fragment, whereas the larger exon 5 with part of the 3'UTR was amplified in 3 overlapping fragments (the first fragment was coamplified with exon 4). The intron-exon boundaries and flanking sequences were included in the regions analyzed. Primers were designed from the published *FGA* sequence (Genbank M64982) and are listed as shown in Table 2.

PCR products were directly sequenced with the primers used for the amplification on a semiautomated sequencer, using standard dye-terminator protocols (PE Biosystems, Foster City, CA). For all patients with apparently

Table 2. Primers used for PCR amplifications (5' > 3')

Target	Forward	Reverse
Exon 1	CAGCCCCACCCTTAGAAAAG	CCTGGGGTCATAAAGCTAAG
Exons 2-3	CCTCTTCTGGCTAACATTGC	CAGGGATATTATGAAGGTATG
Exon 4	CAGCAGCTACTTCAATAACC	GTGCATAACTATCGCCTTCC
Exon 5, 5'	(same as exon 4)	GCGGCATGTCTGTTAATGCC
Exon 5, mid	GATCTTGTGCGAGGGTCATGC	CTCCTAACATAGGTGAGAAG
Exon 5, 3'	CTGGACCTCTGAGAGCTCTG	ATGGCTCTGTACTGTTAGGC

heterozygous changes, the 2 alleles were cloned (TOPO-TA; Invitrogen, Groningen, The Netherlands) and sequenced as above.

Southern blotting

Genomic DNA was digested with *Bam*HI and electrophoresed and blotted by standard techniques. Filters were hybridized with the pAFS1 probe as previously described.⁶

Haplotype analysis

Microsatellite analyses were performed as previously described.⁷ The locus order and genetic distances in centimorgans were: *D4S3021*–(0.0)–*fibrinogen* (*FGAi3/FGB*)–(0.6)–*D4S2999* (according to the Marshfield sex-averaged genetic map, <http://www.marshmed.org>). Because parental chromosomes were unavailable for analysis, allele phases were inferred to determine the minimal number of different chromosomes in the patient collection; the real number was possibly higher.

Screening of normal alleles

Ninety-five DNA samples from anonymous European control subjects were screened for the IVS1 + 3 A > G mutation by PCR amplification of *FGA* exon 1 followed by nonradioactive, single-stranded conformation analysis using GeneGel Excel gels (Pharmacia Biotech, St Albans, UK) as previously described.¹²

Results

Data from 13 unrelated patients with afibrinogenemia were collected for this study. For all these patients, plasma fibrinogen was undetectable by functional and immunologic assays, and the prothrombin, activated partial thromboplastin, and thrombin time tests were unclottable in all patients. Factor V, factor VII, and factor X were measured in all patients and were within normal ranges. In the 7 patients tested, factors VIII, IX, and XI were within normal ranges. Furthermore, for some patients, inhibitors (antithrombin, protein C, and protein S), factor V Leiden, and prothrombin G20210A mutations were assayed, and all results were normal.

The *FGA* gene was screened for mutations by PCR amplification of the entire coding region of the α isoform, and this was followed by DNA sequencing. Heterozygous PCR products were cloned, and the 2 alleles were sequenced. Causative mutations were identified in 22 of 26 disease alleles. Interestingly, the mutations were identified in all patients of Caucasian origin but not in the 2 non-Caucasian patients (Table 1 and Figure 1).

Deletion of 11 kb

We had previously predicted that the originally reported 11-kb *FGA* deletion would be a relatively common recurrent mutation, and we therefore wanted to determine its prevalence in this patient group. The deletion was excluded in patients A5, A6, A8, and A12 because of heterozygosity for the *FGAi3* polymorphism, which is situated within the deletion in intron 3, and in patient B3, a compound heterozygote for 2 different mutations (Table 1). For the remaining

Table 1. Patients with afibrinogenemia investigated in this study

Patient	Origin (consanguinity)	Age at Diagnosis	Mutation	Haplotypes
A2	France (NC)	Birth	IVS4 + 1 G > T	1-5-2
			IVS4 + 1 G > T	2-5-2
A4	Puerto Rico (C)	7 months	Not found	1-1-3
				1-1-3
A5	France (?)	Birth	IVS4 + 1 G > T	1-3-2
			IVS4 + 1 G > T	1-4-1
A6	France (NC)	1 year	IVS4 + 1 G > T	1-5-2
			IVS4 + 1 G > T	2-4-2
A7	France (NC)	10 months	IVS4 + 1 G > T	2-5-2
			G316X (exon 5)	2-6-2
A8	Belgium (NC)	5 years	34insC (exon 1)	2-5-2
			3121delAA (exon 4)	2-2-2
A9	Belgium (C)	2 years	IVS4 + 1 G > T	1-4-1
			IVS4 + 1 G > T	1-4-1
A10	France (NC)	17 years*	IVS4 + 1 G > T	1-5-1
			IVS1 + 3 A > G	3-3-1
A12	France (NC)	5 years	3121delAA (exon 4)	2-2-?
			W334X (exon 5)	2-5-?
B1	France (?)	Birth	IVS4 + 1 G > T	2-3-1
			IVS4 + 1 G > T	2-3-1
B2	Pakistan (C)	17 months	Not found	1-3-1
				1-3-1
B3	United States (?)	Birth	IVS4 + 1 G > T	2-4-2
			4329delC (exon 5)	2-4-1
B4	United States (?)	Birth	IVS4 + 1 G > T	2-5-2
			11-kb deletion	3-del-1

*This female patient was considered to have severe hemophilia from an early age; the formal diagnosis of afibrinogenemia was not made until she was 17 years old. Haplotypes were defined with the 3 closely linked markers, *D4S3021*, *FGAi3*, and *D4S2999*. Because analysis of the parental chromosomes was not possible, allele phases were inferred to determine the minimum number of different chromosomes in the patient sample. C, consanguineous; NC, not consanguineous; ?, unknown status.

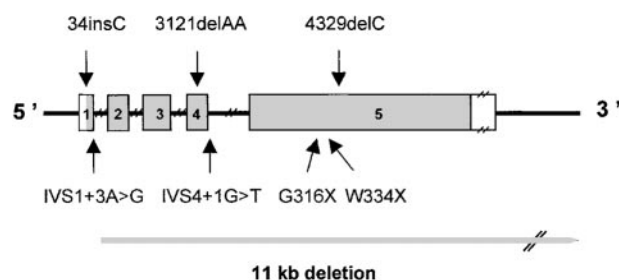


Figure 1. Schematic representation of the *FGA* gene showing mutations causing congenital afibrinogenemia. Exons are numbered and are drawn to scale; the shaded portions represent the coding region. Introns are indicated by narrow lines and are not to scale.

patients, Southern blot analysis was performed as previously described. The patient from the United States (B4) was identified as a carrier of the deletion (Figure 2), confirming its recurrent nature.⁷

Splice site mutations

Five patients were homozygous for a previously undescribed splice site mutation, IVS4 + 1 G > T (A2, A5, A6, A9, and B1), and 4 were compound heterozygotes (A7, A10, B3, and B4; Table 1, Figures 1 and 2).

The IVS4 + 1 G > T represents 54% (14 of 26) of the alleles in this study or 48% of all the afibrinogenemia mutations described to date (including our previous study⁶).

Haplotypes were obtained for 3 microsatellite markers within or closely flanking the fibrinogen gene cluster. Because the parental DNA was unavailable, allele phases could not be determined; remarkably, however, the IVS4 + 1 mutation was found on at least 8 discrete haplotypes (for the 3 markers used; Table 1). Significantly, the mutation was associated with 3 different alleles just for the intragenic *FGA* intron 3 microsatellite marker (arbitrarily numbered 3, 4, and 5; Table 1).

In 1 patient (A10) who was heterozygous for the IVS4 + 1 mutation, the only other change identified by sequencing of the *FGA* gene was an A > G mutation in the intron 1 donor site (IVS1 + 3 A > G). Although the donor site +3 position is not highly conserved in splice sites, this mutation was not found in 190 normal alleles (or in any of the other patients in this study). We predicted that the IVS1 + 3 mutation was the second causative mutation in this patient (see "Discussion").

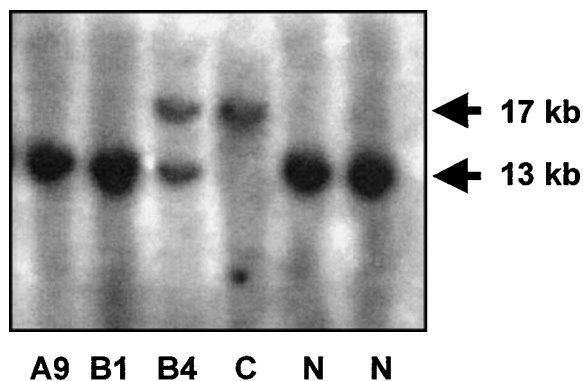


Figure 2. Southern blot diagnosis of the *FGA* 11-kb deletion. The normal fragment detected by probe pAFS1 measures 13 kb, the deleted allele is detected as a 17-kb fragment because 2 *Bam*HI sites are eliminated. The results for 3 patients are shown. C, homozygous deleted control; N, normal control.

Deletions and insertions

One patient (A8) was found to be a compound heterozygote for 2 frameshift mutations in *FGA*. The first mutation, 34insC (numbered according to Genbank M64982), was a 1-bp insertion in the second codon of exon 1 that led to an in-frame TAG stop codon in exon 2. The second mutation, 3121delAA, was a 2-bp deletion in exon 4 that led to an in-frame TAG stop codon 15 codons downstream, in the same exon. Another patient (A12) was also a compound heterozygote for 3121delAA. A third frameshift mutation in exon 5, 4329delC, was identified in patient B3. This deletion generated an in-frame TAA stop codon 69 codons downstream.

Nonsense mutations

Two nonsense mutations were identified in exon 5, both in compound heterozygosity: G316X (patient A7, GGA > TGA) and W334X (patient A12, TGG > TGA).

Discussion

In this study, we analyzed the *FGA* gene in 13 unrelated patients with congenital afibrinogenemia from Puerto Rico, France, Pakistan, Belgium, and the United States to identify the causative mutations in affected persons and to determine the prevalence of the 11-kb *FGA* deletion. Including the 3 alleles in the 4 affected family members described in our original report,⁶ we have now studied a total of 29 alleles in 17 patients and have identified 8 different *FGA* mutations that account for 86% (25 of 29) of the afibrinogenemia alleles.

In our previous studies, we described 4 affected persons, all homozygous for an 11-kb deletion that was identical to the base pair.^{6,7} Surprisingly, haplotype analysis suggested that the deletion occurred on 2 if not 3 discrete ancestral chromosomes, perhaps as a result of an illegitimate recombination mediated by direct and indirect repeats in the breakpoint region. The identification here of 1 additional allele bearing the 11-kb deletion, in an American patient unrelated to the Swiss family previously described, confirms that the *FGA* 11-kb deletion is indeed recurrent.

The novel IVS4 + 1 G > T mutation we report here, at the invariant GT dinucleotide of the intron 4 donor splice site, also appeared to be recurrent and is the most common known cause of congenital afibrinogenemia, accounting for 14 of the 29 (48%) mutant alleles we studied. Alternatively, it remains possible that these 2 mutations were not recurrent but were inherited from common ancestors; it could be predicted that they are very ancient mutations because of their association with numerous haplotypes.

Although it proved impossible to determine the effect of this mutation in patients' leukocyte RNA (the levels of illegitimate expression were too low to detect), the fact that these patients have clinical afibrinogenemia and not severe hypofibrinogenemia implies that essentially no normal splicing occurs at this site. Among mutations of the consensus dinucleotide splice sites, those at the +1 position of the donor 5' site are the most frequent.¹³ In most patients, they lead to skipping of the exon immediately 5' of the mutation.

We performed computer splice prediction analysis of the region around the IVS4 donor site with the program Spliceview,¹⁴ which predicts splice-site efficiencies taking into account consensus sequence homology and the mutual dependency of nucleotides (<http://www.itba.mi.cnr.it/webgene/>). In the normal sequence, 2 distinct predicted donor sites were detected, the first "physiological," and the second 5 bases downstream that resulted in a

frameshift mutation. In the presence of the IVS4 + 1 G > T mutation, the physiological site is no longer detected by the analysis. We therefore predict that the common mutation leads to aberrant usage of the alternative site and to a consequent frameshift mutation in 100% of the transcripts.

The second donor splice site mutation, IVS1 + 3 A > G, was present in only 1 patient, in compound heterozygosity with the IVS4 + 1 G > T mutation. It was the only other sequence variant identified in this patient. We excluded the possibility that this was a common polymorphism in the human *FGA* gene by screening 190 alleles from normal persons; no other carrier was found. Splice-view analysis of the normal sequence around the IVS1 donor site predicts a single site; however, in the mutant IVS1 + 3 G sequence, no donor site is identified. Furthermore, although the consensus donor splice site has either A or G at this position,¹⁵ 11 disease-causing splice site + 3 A > G mutations have been reported to date; in 10 of these, aberrant splicing was proven.¹⁶ Consequently, it is probable that this represents the second pathogenic mutation in patient A10, though the undetectable levels of *FGA* mRNA in blood leukocytes made it impossible to evaluate its *in vivo* effect.

We have also identified 3 novel frameshift mutations, all of which are predicted to result in premature termination of translation because of the creation of in-frame stop codons and 2 nonsense mutations.

Although all the mutations were identified in Caucasian patients, we did not find any *FGA* mutations in patients A4 and B2 (of Puerto Rican and Pakistani origins, respectively). Both these patients are consanguineous and homozygous for the 3 polymorphic markers used. It is likely that they have mutations in the closely linked *FGG* or *FGB* genes, which were not analyzed in this study. Missense mutations in *FGB* have recently been identified in 2 patients with afibrinogenemia.¹⁷

We have now identified 8 different mutations in the fibrinogen

α gene, accounting for the majority of afibrinogenemia alleles investigated, though intuitively it could be expected that all the fibrinogen genes would be equally implicated. All the mutations were predicted to lead to premature termination (Figure 1). Four such mutations have been previously described in *FGA* exon 5, in association with either dysfibrinogenemia or severe hypofibrinogenemia. Three of these are situated much closer to the carboxy-terminus than the mutations we describe here, leading to termination after amino acid 451 or higher.¹⁸⁻²⁰ Fibrinogen Otago,²¹ however, was associated with a homozygous frameshift mutation, leading to the truncation of fibrinogen α at amino acid 270, which is proximal to 3 of the afibrinogenemia mutations we describe. In our patients, these mutations were present in compound heterozygosity associated with more amino-terminal truncation mutations (Table 1). It is possible that the more distal truncation mutations are functionally less severe and permit the secretion of minute quantities of fibrinogen, which, in homozygosity or in compound heterozygosity with less severe *FGA* mutations, result in hypofibrinogenemia rather than afibrinogenemia. If this is the case, we expect to find some of the mutations we described here in patients with less severe fibrinogen disorders in association with less severe mutations. The characterization of these new mutations will facilitate diagnosis of these disorders and permit prenatal diagnosis for families who so desire.

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