ARTICLE

Congenital afibrinogenaemia caused by uniparental isodisomy of chromosome 4 containing a novel 15-kb deletion involving fibrinogen A α -chain gene

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Among rare inherited deficiencies of coagulation factors, congenital afibrinogenaemia is characterised by the lack of fibrinogen in plasma. In the last few years, several genetic defects underlying afibrinogenaemia (mostly point mutations) have been described in the fibrinogen gene cluster. In this study, the molecular basis responsible for afibrinogenaemia in a Thai proband was defined. Point mutation screening was accomplished by directly sequencing the three fibrinogen genes. The impossibility to amplify fibrinogen A α -chain gene (*FGA*) exons 5 and 6 suggested the presence of a homozygous deletion. A specific long-range PCR assay enabled the identification of a novel 15-kb deletion, representing the largest afibrinogenaemia-causing deletion described so far. Direct sequencing of the deletion junction allowed mapping of the breakpoints in *FGA* intron 4 and in the intergenic region between A α - and B β -chain genes. Since the mutation was inherited only from the mother and nonpaternity was ruled out, a maternal uniparental disomy (UPD) was hypothesised. UPD test, carried out with markers covering the whole chromosome 4, revealed that maternal isodisomy was responsible for homozygosity of the 15-kb deletion in the proband. The apparently normal phenotype of the proband, except for afibrinogenaemia, suggests that UPD for chromosome 4 is clinically silent. This represents the first case of a documented complete isodisomy of chromosome 4 causing the phenotypic expression of a recessive disorder. *In silico* analyses of the regions surrounding the breakpoints suggested that the 15-kb deletion might have originated from an inappropriate repair of a double-strand break by the nonhomologous end joining mechanism.

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Introduction

Circulating fibrinogen is a glycoprotein involved in the final step of the coagulation pathway as a precursor of fibrin monomers that participate in the formation of the haemostatic plug. Fibrinogen is synthesised in hepatocytes as a homodimer composed of pairs of A α , B β , and γ chains.¹ The fibrinogen cluster, comprising the *FGA* (A α chain), *FGB* (B β chain), and *FGG* (γ chain) genes, spans approximately 50 kb at the 4q31.3–4q32.1 chromo-

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somal region.² The three genes are arranged in the *FGG*, *FGA*, and *FGB* order, with *FGB* in opposite transcriptional orientation.³

Congenital afibrinogenaemia (MIM #202400), one of the rarest clotting disorders, shows an autosomal recessive inheritance and is characterised by a severe plasma fibrinogen deficiency and a pattern of haemorrhagic manifestations of variable severity.⁴

The majority of gene alterations underlying congenital afibrinogenaemia are point mutations (two small insertions, six small deletions, four missense, eight splicing, and 11 nonsense mutations), almost equally spread over the fibrinogen genes.^{5–13} Only two large deletions, an 11-kb deletion representing the first causative deletion identified in afibrinogenaemia¹⁴ and a very recently reported 1238-bp deletion,¹³ have been identified and both are located in *FGA*.

In contrast to classical Mendelian inheritance, uniparental disomy (UPD), that is, the inheritance of a pair of homologous chromosomes from one parent, can occur as both hetero- or isodisomy and can result in clinical conditions either by producing homozygosity for recessive mutations or as a consequence of aberrant patterns of imprinting. To date, out of the 47 theoretical possibilities of UPD for entire chromosomes, 32 have been observed (E Engel, personal communication). Only five cases of maternal UPD for chromosome 4 have been reported so far. Three were partial UPDs, involving 4q21–35 in a Japanese man affected by abetalipoproteinaemia,¹⁵ 4p13–16 in a patient affected by Ellis-van Creveld syndrome,¹⁶ and 4p15–16 in a child with trisomy 21;¹⁷ one was a complete UPD in a normally developed foetus who died in the uterus probably because of the high level of mosaicism for trisomy 4 found in placental trophoblast,¹⁸ and one was a case of isochromosome 4 (46, -4, -4, +i4q, +i4p) in a woman with multiple miscarriages.¹⁹

This study describes the first case of congenital afibrinogenaemia caused by uniparental isodisomy (iUPD) of chromosome 4 containing a novel homozygous large deletion (approximately 15 kb). It represents the largest afibrinogenaemia-causing deletion identified so far and involves the 3' half of *FGA* and almost the whole *FGA*–*FGB* intergenic region. Sequence analysis of the deleted allele not only enabled the determination of the deletion junction and of the exact extent of the deletion but also showed the presence of short direct repeats flanking the breakpoints, possibly involved in the molecular mechanism underlying the mutation.

Materials and methods

Informed consent was acquired from all family members prior to their enrolment in the study.

Laboratory studies

Plasma fibrinogen concentration was determined by a functional assay based on fibrin polymerisation time²⁰ and by an enzyme-linked immunosorbent assay,²¹ whose sensitivities were 5 and 0.0005 mg/dl, respectively (normal ranges for both assays: 160–400 mg/dl). Coagulation tests, including activated partial thromboplastin time, prothrombin time, and thrombin time, were performed by routine methods (reference intervals: 24–38", 10.5–12", and 15–18", respectively).

Mutation analysis

Genomic DNA was purified from peripheral blood according to standard protocols. Point mutation detection was performed by directly sequencing PCR products encompassing all coding regions, exon–intron junctions, and about 300 bp of the promoter regions of *FGA*, *FGB*, and *FGG*. PCR amplifications were performed on 100–400 ng of genomic DNA, as described.⁸ The amplified fragments were purified by ammonium-acetate precipitation and directly sequenced on both strands by using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and the same primers used in amplification reactions except for the long *FGA* exons 5 and 6, which required additional internal primers. Sequencing products were analysed on an ABI-3100 Genetic Analyser (Applied Biosystems). Mutation detection was accomplished by means of Factura and Sequence navigator packages (Applied Biosystems) and using, as reference, sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/>) under accession numbers: M64982, M64983, M10014, U36478, AF229198, and AC107385.

Long-range PCRs

Long-range PCRs, encompassing the deleted region, were carried out by using the Expand 20 kb^{plus} PCR System kit (Roche, Basel, Switzerland) and the primer couple *FGA*-Ex4-In4-F (5'-GACTGGAGGTAAGTATGTGGCTGTG-3') and *FGB*-Ex8-F (5'-CTAAAGAAGACGGTGGTGGATGGTG-3') (Invitrogen, Carlsbad, CA, USA). Reactions were performed on 250–500 ng of genomic DNA of the proband and of the available family members or on 250 ng of human control genomic DNA provided with the kit, according to the manufacturer's instructions, in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA). Amplification conditions were: an initial denaturation step at 92°C for 2', 10 cycles of denaturation at 92°C for 10", annealing at 61°C for 30", and polymerisation at 68°C for 20', followed by 20 cycles under the same conditions with an incremental lengthening of the polymerisation time of 10" each cycle, and a final extension step at 68°C for 7'.

Microsatellite analyses

Paternity test was accomplished by PCR amplification of 10 short tandem repeat (STR) markers from chromosomes 2, 3,

4, 8, 11, 12, 16, 18, 19, and 21 using the AmpFISTRSMG-Pluskit (Applied Biosystems), according to the manufacturer's instructions.

UPD test was performed by PCR amplification of 22 highly polymorphic STRs, selected from the Génethon human linkage map, using the ABI PRISM Linkage Mapping Set MD10 (Applied Biosystems) containing pairs of labelled forward primer and unlabelled reverse primer. PCRs were performed on 60 ng of genomic DNA in a 15 µl reaction volume containing 1 × reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM MgCl₂, and 0.1% Triton X-100), 2 mM MgCl₂, 200 µM deoxynucleoside triphosphate, 0.6 µM of primer mix, and 0.6 U Taq DNA Polymerase (Promega, Milan, Italy) in a PTC-100 thermal cycler (MJ Research). Thermal conditions were: an initial denaturation step at 95°C for 3', 35 cycles of denaturation at 95°C for 30", annealing at 55°C for 30", and polymerisation at 72°C for 30", followed by a final extension step at 72°C for 10'. Amplification of the polymorphic locus *FGA*-i3 was performed as described.²²

Genotyping was accomplished by using Genescan 3.1 software (Applied Biosystems).

Computer-assisted analyses

Analysis of interspersed repetitive elements was performed by using the public Blat software at the UCSC Genome Browser.² Searches for inverted repeats and matrix-scaffold attachment regions were accomplished by using Einverted and Marscan programs, respectively, freely available at the EMBOSS web site (<http://bioinfo.pbi.nrc.ca:8090/EMBOSS/index.html>).

Results

Clinical details of the analysed family

A young Thai girl born by normal delivery in 1999 was analysed. The proband suffered from two episodes of prolonged bleeding that required hospitalisation and blood transfusions: a spontaneous blood oozing from the umbilical cord stump at 4 days after birth and a post-traumatic haemorrhage of the scalp as a consequence of a cat bite wound at the age of 1 year. Diagnosis of afibrinogenaemia was made after laboratory measurements (performed twice at a 1-year interval) that revealed unmeasurable levels of functional and immunoreactive plasma fibrinogen (Figure 1) and infinite activated partial thromboplastin time, prothrombin time, or thrombin time.

No history of bleeding episodes was reported by the 40-year-old proband's father, the 34-year-old proband's mother and by the 13- and the 10-year-old proband's sisters (individuals II-1 and II-2, respectively, Figure 1). Coagulation tests were performed on all available family members. Slightly reduced (137 mg/dl) and low borderline (185 mg/dl) levels of functional fibrinogen were measured in the proband's mother and sister II-2, respectively

Figure 1 Pedigree of the Thai family. Functional and immunoreactive plasma fibrinogen levels (mg/dl) are reported in this order below each symbol. The arrow indicates the proband.

(Figure 1), while the other tests were within the range of normality.

Point mutation screening

The mutational screening of the proband was performed by PCR amplifications of *FGA*, *FGB*, and *FGG* exons (including splicing junctions) and promoter regions, followed by direct sequencing. The expected PCR products were obtained for all amplified fragments, except those corresponding to exons 5 and 6 of *FGA*. To exclude the possibility that the failed amplifications were due to poor integrity of the genomic DNA or to misannealing of the primers, further PCR assays were performed increasing the amount of genomic DNA (up to 400 ng) in the reaction mixture and using different primer couples. In all cases, no amplification was obtained. These results suggested the presence of a homozygous deletion, involving at least the unamplifiable exons. This hypothesis was further supported by the successful amplification of *FGA* exons 5 and 6 from the genomic DNA of the proband's parents and sisters and by the lack of any novel or previously reported point mutation in the sequenced regions of the fibrinogen cluster of the proband.

Identification of the 15-kb deletion

To confirm the deletion and to determine its extension, a long-range PCR assay was performed on the proband's genomic DNA with primers *FGA*-Ex4-In4-F (located across *FGA* exon 4-intron 4 junction, at nucleotide position 3193–3217, according to GenBank accession number M64982) and *FGB*-Ex8-F (located in *FGB* exon 8, at nucleotide position 7900–7924, according to GenBank accession number M64983) (Figure 2a). A single amplification product of about 1.8 kb, much shorter than the expected wild-type one (about 17 kb), was visualised after agarose-gel electrophoresis (Figure 2a and b, upper panel), confirming the presence of an about 15-kb deletion. The same long-range amplification reaction was carried out on

Figure 2 Detection of the 15-kb deletion by the long-range PCR assay. (a) Schematic representation of part of the fibrinogen cluster. Afibrinogenaemia-causing large deletions identified so far in the fibrinogen cluster are indicated. Boxes (numbered) and lines represent exons and intronic/intergenic regions, respectively, and are drawn to scale. The two parallel slanted lines indicate break in the scale. White and grey arrows indicate transcriptional orientation of *FGB* and *FGA*, respectively, while black arrows show the position of the primer couple used in the long-range PCR assay. Sizes of wild-type and mutant PCR fragments are also reported. (b) Detection by agarose (1%) gel electrophoresis of the long-range PCR products (upper panel) and of the amplicons corresponding to *FGA* exon 5 (lower panel), both amplified from genomic DNAs of the analysed family members and of a control individual. λ -PstI and λ -HindIII are DNA molecular weight markers. The deduced genotype of each analysed individual is reported below the corresponding lane. 'Del' denotes the 15-kb deleted allele, and '+' indicates the wild-type one.

genomic DNA from all family members and on a control genomic DNA. The abnormal 1.8-kb band was amplified from the proband's mother and sister II-2, while it was undetectable in proband's father, sister II-1, and in the control DNA (Figure 2b, upper panel). Taking into account the considerable difference in length between amplicons from wild-type and mutant alleles (17 vs 1.8 kb), which dramatically favours the shorter fragment amplification, and considering that *FGA* exons 5 and 6 were successfully amplified from the DNA of the proband's parents and sisters (Figure 2b, lower panel), the identified large deletion was concluded to be present in the homozygous state in the proband and in the heterozygous state in both proband's mother and sister II-2, while the father and individual II-1 were not carriers of the deleted allele (Figure 2b).

UPD analysis

Since the proband's father was proven to be homozygous for the wild-type allele at the deletion site, despite autosomal recessive inheritance of afibrinogenaemia, indicating this individual as an obligate heterozygote, a

paternity test was carried out to rule out a possible nonpaternity. All 10 typed markers (on 10 different chromosomes) were consistent with paternal inheritance, with the only exception of the intragenic *FGA*-i3 marker, located in intron 3 of the fibrinogen α -chain gene. Mendelian segregation of the *FGA*-i3 marker was found in the proband's sisters. These data were suggestive of a maternal disomy for chromosome 4. In order to verify this hypothesis, genotype analysis for STR markers spread over the entire chromosome 4 was performed in the proband and her parents. Out of 23 analysed STRs, 14 were informative and indicated that the proband possesses only the mother's alleles (Figure 3). Moreover, all the analysed STRs showed reduction to homozygosity in the proband (Figure 3). Taken together, these results confirmed maternal isodisomy of chromosome 4.

Further characterisation of the deletion

In order to map the deletion breakpoints, the 1.8-kb product was directly sequenced. Comparison between this sequence and the RP11-158C21 genomic clone (GenBank, Accession Number AC107385), containing the fibrinogen

Figure 3 Genotype analysis for chromosome 4 markers in the proband and her parents. Chromosomal localisation of markers used for genotyping is shown on the ideogram of chromosome 4 (on the left). Genotypes of the analysed individuals (on the right) are shown as recoded alleles, indicated from the smallest to the largest. Boxes shaded in grey indicate evidence for UPD.

cluster, revealed a normal sequence up to nucleotide 369 of *FGA* intron 4, joined to the last 824 nucleotides of *FGA-FGB* intergenic region (located upstream of the *FGB* 3'UTR). The deleted region spanned 15228 nucleotides (position 93571–108798, according to the reverse complement of the database sequence AC107385) (Figure 4a and b).

In an attempt to identify *cis* elements putatively involved in the deletion mechanism, *in silico* analyses of the regions surrounding the breakpoints were performed.

The wild-type sequence showed the presence of 4-bp (ACAG) direct repeats at both cleavage sites (Figure 4b). Considering that in the deleted allele only one repeat copy is retained, the precise location of the 5' and 3' breakpoints cannot be unambiguously determined, and may therefore

Figure 4 Identification and characterisation of the 15-kb deletion breakpoints. (a) Sequence electropherogram of the region of the aberrant 1.8-kb band corresponding to the deletion junction. The nucleotide position of the deleted region is also indicated. Sequences located 5' and 3' to the deletion junction correspond to *FGA* intron 4 and *FGA-FGB* intergenic region, as indicated below the electropherogram. (b) Characterisation of the sequence elements located around the deletion breakpoints. Part of the genomic region comprised between nucleotides 93457 and 108908 (according to the reverse complement of the database sequence AC107385) is reported. The deleted region is underlined. Direct repeats are in bold and adenine-thymine rich regions are in italic. Asterisks above and below the sequence indicate the consensus sequences for Topo I cleavage sites located on the forward and on the reverse strand, respectively. Black and grey arrows mark direct and inverted repeats, respectively.

range between positions 93567–93571 and 108794–108798, respectively (Figure 4b).

Further examination of the sequences immediately surrounding the two breakpoints revealed the presence of multiple trinucleotide consensus sequences for Topoisomerase I (Topo I) cleavage sites. In particular, the 5' breakpoint is surrounded by eight Topo I putative cleavage sites (GAT, AAT, and two CAT in the forward strand; two AAT, GTT, and CAT in the reverse strand), while seven Topo I cleavage sites (CAT and two AAT in the forward strand; CAT, AAT, GTC, and CTT in the reverse strand) are located near the 3' breakpoint (Figure 4b). Furthermore, adenine-thymine-rich regions, localised just before (AATTAAA) and after (AAATTAAT) the deletion junction, were found (Figure 4a and b). Taken together, these regions contain both a 7-bp 'symmetric element' (AATTAAA/AAATTAAT) and a 5-bp inverted repeat (ATTAA/TTAAT) (Figure 4a and b).

Figure 5 Elements identified by computer-assisted analyses in the regions surrounding the breakpoints and in the entire 15-kb deleted region. Numbering refers to the 15-kb deleted region; arrowheads indicate the breakpoints. Distances between elements are drawn to scale. White and hatched boxes indicate LINE and SINE elements, respectively. Hairpin loops represent the potentially secondary structures with the longer stem and the higher percentage of matches among inverted repeats. Hairpin lengths (in bold) as well as percentages of identity and number of gaps between inverted repeats (in parentheses) are reported beside each stem. The grey arrows indicate the orientation of the sequences composing the bipartite MRS element.

Regions encompassing the deletion were also analysed for the presence of interspersed repetitive elements. To this aim, sequences of *FGA* and of *FGA-FGB* intergenic region (GenBank, accession numbers M64982 and AC107385, respectively) were submitted to the Blat software. Neither the 5' nor the 3' breakpoints were found to lie within a repetitive element; however, both occurred between a short interspersed element (SINE) and a long interspersed element (LINE). In particular, an MIR family element (MIRb) and an L2 repeat (L2) were located 122 bp upstream and 1609 bp downstream of the 5' breakpoint, respectively, while an L1 repeat (L1MA10) and an Alu family member (AluSg) were found 323 bp upstream and 419 bp downstream of the 3' breakpoint, respectively (Figure 5).

In addition, the presence of inverted repeats and of matrix-scaffold attachment regions (MARs/SARs) was searched by computer-assisted analyses in the wild-type region corresponding to the 15-kb deletion. Five inverted repeats, which can form hairpin structures ranging from 22 to 275 bp (percentage of identity varying from 75 to 90%, with 0 to 6 gaps), were identified (Figure 5). Moreover, an MAR/SAR recognition signature (MRS) composed of two individual sequences (AATAATAA and AAAATAAT-TAAGTTT), located at 5-bp distance from each other, was found. This bipartite sequence element is localised almost exactly in the middle of the deleted region (Figure 5).

Discussion

Since point mutations in the fibrinogen cluster account for about 82% of afibrinogenaemia-causing alleles, the search

for the gene alteration underlying afibrinogenaemia in the analysed proband was initially based on PCR amplification followed by direct sequencing. The absence of nucleotide variations and the impossibility of amplifying *FGA* exons 5 and 6 suggested the presence of a large deletion. A specific long-PCR protocol was set up and enabled the identification of a novel 15228-bp deletion that removes about 53% of *FGA* and 93% of *FGA-FGB* intergenic region.

As in the analysed family a non-Mendelian segregation of the mutant allele was observed, after confirmation of the pedigree structure by paternity test, UPD was considered as a probable cause of the homozygosity for the identified recessive mutation. Indeed, numerous cases of UPD have been identified through homozygosity for recessive mutations (eg cases of cystic fibrosis, haemophilia A, and spinal muscular atrophy III).²³

Microsatellite analysis in the proband and her parents disclosed a maternal uniparental isodisomy of chromosome 4, representing the first case of iUPD in an afibrinogenemic patient. Even though several mechanisms (gametic complementation, trisomy rescue, and compensatory UPD) could be responsible for UPD,²⁴ the finding of homozygous markers throughout chromosome 4 of the analysed proband represents an indirect evidence for a postzygotic somatic event, possibly due to nondisjunction in the paternal meiosis (nullisomic gamete) followed by mitotic duplication of the maternal homologue in the monosomic zygote (compensatory UPD). With respect to chromosome 4, only one case of UPD for maternal isochromosome 4p and 4q has been described in a living patient, a woman who suffered multiple early miscarriages and, besides being the tallest among her siblings, was

phenotypically normal.¹⁹ Our proband appears normal except for manifestations of afibrinogenaemia; a comparison with the height of her two sisters is not yet feasible since the proband is only 5 years old. These data confirm that there are probably no imprinted genes on chromosome 4 with a major effect on phenotype. Nevertheless, since no case of paternal UPD for chromosome 4 has been reported to date, the possibility that some regions of chromosome 4 could be paternally inactivated cannot be excluded.

Unlike other diseases, large deletions are not frequent in afibrinogenaemia, since only two shorter deletions (1238 bp and 11 kb) have been described so far.^{13,14} Interestingly, all deletions involve *FGA* and the deletion breakpoints have similar locations. In particular, the 5' breakpoint of the 15-kb deletion is located in the *FGA* intron 4, like the 3' end of the 1238-bp deletion, while both the 3' breakpoints of the 15- and of the 11-kb deletions are localised in the *FGA*–*FGB* intergenic region (Figure 2a). These data suggest that these genomic regions could be particularly prone to breakage events.

Despite the frequent occurrence (72.5%) of deletions in genomic rearrangements,²⁵ the mechanisms by which these events occur are not yet well understood. The major insights into the molecular variables (ie local primary and secondary DNA structures) predisposing to deletion came from studies on genomic regions characterised by a high recombination rate.²⁶

In the attempt to elucidate the mechanism(s) of the naturally occurring 15-kb deletion, searches for specific *cis* elements were performed. No sequences with extensive homology (eg interspersed repetitive elements, like SINE and LINE), which could play a role in homologous recombination (a frequent mechanism of gene deletion found in many genetic diseases^{27,28}), were identified across the cleavage sites. Conversely, short (4-bp long) homology sequences flanking the breakpoints were detected. This molecular configuration can support several hypotheses on the mechanisms underlying the formation of the identified deletion. Probably, the 4-bp direct repeats are not long enough to mediate unequal crossing over via homologous recombination. Nevertheless, as proposed for other deletions,²⁹ both the presence of direct repeats and the deletion of an entire repeat copy plus all the intervening sequence might be the result of a slipped mispairing during DNA replication. Besides this model, an inappropriate repair of double-strand breaks (DSBs), which can occur during the exposure to DNA-damaging agents or as a molecular intermediate in several cellular processes, could be postulated as the cause of the 15-kb deletion. In mammalian cells, DSBs are commonly repaired by the nonhomologous end joining (NHEJ) mechanism, which requires little or no sequence homology at the broken DNA ends.³⁰ In the vast majority of studied regions prone to nonhomologous recombination, Topo I cleavage sites, also in association

with A/T rich regions, have been described in the 10-bp vicinity of the breakpoints.³¹ The findings of both features (ie multiple Topo I cleavage sites and runs of adenines and thymines) at the breakpoints of the 15-kb deletion described here further support the NHEJ hypothesis. If the role of Topo I in nonhomologous recombination can be postulated on the basis of its enzymatic activity, the meaning of the A/T rich regions is unclear.³¹ In the present case, the presence of a 'symmetric element' and of an inverted repeat within the A/T rich regions suggests that they could make unusual DNA structures during the NHEJ process. Finally, the observed chromosomal deletion might also be the result of a single-strand annealing (SSA) process, which plays a minor role in the DSBs repair. As NHEJ, SSA relies on the presence of repeated sequences flanking the breaks, even though it acts as a nonconservative repair mechanism by causing the loss of the region between the repeats.³⁰

Whatever mechanism (slippage mispairing, NHEJ, or SSA) is involved in the formation of the identified deletion, it should require bringing together of the two breakpoints. In this respect, the presence in the deleted region of several long inverted repeats, able to form looped secondary structures, and of an MRS element, that anchors the chromatin to the nuclear matrix or scaffold, could help in bringing together the 5' and 3' breakpoints.

The presence of short direct repeats, such as the 4-bp sequences identified during the molecular characterisation of the 15-kb deletion, represents a common feature at the breakpoints of large deletions in the fibrinogen cluster. Indeed, analysis of the breakpoints of the 1238-bp deletion identified the presence of 2-bp direct repeats, whereas 7-bp direct repeats were evidenced in the case of the 11-kb deletion.³² Since direct repeats occur more frequently than would be expected from random breakage and reunion, they probably play an important role in the deletion formation, which could be mediated by a common mechanism. In the case of the 15-kb deletion reported here, available data on molecular features indicate the NHEJ process as the most probable cause of this deletion.

In conclusion, this work reports the only documented case of a maternal iUPD for the entire chromosome 4. This condition caused the anomalous segregation of a novel 15-kb deletion involving the *FGA* gene in a Thai family, leading to the first case of congenital afibrinogenaemia due to uniparental isodisomy.

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References

- Mosesson MW: Fibrinogen structure and fibrin clot assembly. *Semin Thromb Hemost* 1998; **24**: 169–174.
- UCSC Genome Browser, <http://genome.ucsc.edu/>.
- Kant JA, Fornace AJ Jr, Saxe D, Simon MI, McBride OW, Crabtree GR: Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion. *Proc Natl Acad Sci USA* 1985; **82**: 2344–2348.
- Peyvandi F, Duga S, Akhavan S, Mannucci PM: Rare coagulation deficiencies. *Haemophilia* 2002; **8**: 308–321.
- Asselta R, Duga S, Spena S *et al*: Congenital afibrinogenemia: mutations leading to premature termination codons in fibrinogen α -chain gene are not associated with the decay of the mutant mRNAs. *Blood* 2001; **98**: 3685–3692.
- Neerman-Arbez M: The molecular basis of inherited afibrinogenemia. *Thromb Haemost* 2001; **86**: 154–163.
- Asselta R, Spena S, Duga S *et al*: Analysis of Iranian patients allowed the identification of the first truncating mutation in the fibrinogen β -chain gene causing afibrinogenemia. *Haematologica* 2002; **87**: 855–859.
- Spena S, Duga S, Asselta R, Malcovati M, Peyvandi F, Tenchini ML: Congenital afibrinogenemia: first identification of splicing mutations in the fibrinogen β -chain gene causing activation of cryptic splice sites. *Blood* 2002; **100**: 4478–4484.
- Vlietman JJ, Verhage J, Vos HL *et al*: Congenital afibrinogenaemia in a newborn infant due to a novel mutation in the fibrinogen α -chain gene. *Br J Haematol* 2002; **119**: 282–283.
- Neerman-Arbez M, Vu D, Abu-Libdeh B, Bouchardy I, Morris MA: Prenatal diagnosis for congenital afibrinogenemia caused by a novel nonsense mutation in the *FGB* gene in a Palestinian family. *Blood* 2003; **101**: 3492–3494.
- Spena S, Asselta R, Duga S *et al*: Congenital afibrinogenemia: intracellular retention of fibrinogen due to a novel W437G mutation in the fibrinogen β -chain gene. *Biochim Biophys Acta* 2003; **1639**: 87–94.
- Vu D, Bolton-Maggs PH, Parr JR, Morris MA, Neerman-Arbez M: Congenital afibrinogenemia: identification and expression of a missense mutation in *FGB* impairing fibrinogen secretion. *Blood* 2003; **102**: 4413–4415.
- Watanabe K, Shibuya A, Ishii E *et al*: Identification of simultaneous mutation of fibrinogen α chain and protein C genes in a Japanese kindred. *Br J Haematol* 2003; **120**: 101–108.
- Neerman-Arbez M, Honsberger A, Antonarakis SE, Morris MA: Deletion of the fibrinogen alpha-chain gene (*FGA*) causes congenital afibrinogenemia. *J Clin Invest* 1999; **103**: 215–218.
- Yang XP, Inazu A, Yagi K, Kajinami K, Koizumi J, Mabuchi H: Abetalipoproteinemia caused by maternal isodisomy of chromosome 4q containing an intron 9 splice acceptor mutation in the microsomal triglyceride transfer protein gene. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1950–1955.
- Tompson SW, Ruiz-Perez VL, Wright MJ, Goodship JA: Ellis-van Creveld syndrome resulting from segmental uniparental disomy of chromosome 4 (Abstract). *J Med Genet* 2001; **38**: E18.
- Starke H, Mitulla B, Nietzel A *et al*: First patient with trisomy 21 accompanied by an additional der(4)(p11→q11:) plus partial uniparental disomy 4p15–16. *Am J Med Genet* 2003; **116A**: 26–30.
- Kuchinka BD, Barrett IJ, Moya G *et al*: Two cases of confined placental mosaicism for chromosome 4, including one with maternal uniparental disomy. *Prenatal Diagn* 2001; **21**: 36–39.
- Lindenbaum RH, Woods CG, Norbury CG, Povey S, Ryslecl G: An individual with maternal disomy of chromosome 4 and iso (4p), iso (4q) (Abstract). *Am J Med Genet* 1991; **49** (Supplement 285): 1582.
- Clauss A: Gerinnungsphysiologische schnell-methode zur bestimmung des fibrinogens. *Acta Haematol* 1957; **17**: 237–246.
- Cattaneo M, Bettega D, Lombardi R, Lecchi A, Mannucci PM: Sustained correction of the bleeding time in an afibrinogenaemic patient after infusion of fresh frozen plasma. *Br J Haematol* 1992; **82**: 388–390.
- Duga S, Asselta R, Santagostino E *et al*: Missense mutations in the human fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. *Blood* 2000; **95**: 1336–1341.
- Engel E: Invited Editorial: Uniparental disomies in unselected populations. *Am J Hum Genet* 1999; **63**: 962–966.
- Robinson WP: Mechanisms leading to uniparental disomy and their clinical consequences. *BioEssays* 2000; **22**: 452–459.
- Human Gene Mutation Database, <http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>.
- Toffolatti L, Cardazzo B, Nobile C *et al*: Investigating the mechanism of chromosomal deletion: characterization of 39 deletion breakpoints in introns 47 and 48 of the human dystrophin gene. *Genomics* 2002; **80**: 523–530.
- Labuda D, Zietkiwicz E, Mitchell GA: Alu elements as a source of genome variation: deleterious effects and evolutionary novelties; in Maraia RJ (ed): *The impact of short interspersed elements (SINES) on the host genome*. Austin, Heidelberg: Landes company, TX/ Springer-Verlag; 1995, pp 1–24.
- Burwinkel B, Kilimann MW: Unequal homologous recombination between LINE-1 elements as a mutational mechanism in human genetic disease. *J Mol Biol* 1998; **277**: 513–517.
- Luzi P, Rafi MA, Wenger DA: Characterization of the large deletion in the *GALC* gene found in patients with Krabbe disease. *Hum Mol Genet* 1995; **4**: 2335–2338.
- Pastink A, Eeken JC, Lohman PH: Genomic integrity and the repair of double-strand DNA breaks. *Mutat Res* 2001; **480–481**: 37–50.
- Konopka AK: Compilation of DNA strand exchange sites for non-homologous recombination in somatic cells. *Nucleic Acids Res* 1988; **16**: 1739–1758.
- Neerman-Arbez M, Antonarakis SE, Honsberger A, Morris MA: The 11 kb *FGA* deletion responsible for congenital afibrinogenaemia is mediated by a short direct repeat in the fibrinogen gene cluster. *Eur J Hum Genet* 1999; **7**: 897–902.