Unusual (CGG)_n expansion and recombination in a family with fragile X and DiGeorge syndrome

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Received 6 October 1994
Revised version
accepted for publication
24 November 1994

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Abstract

In a fragile X family referred for prenatal diagnosis, the female fetus did not inherit the full fragile X mutation from her mother, but an unexpected expansion within the normal range of CGG repeats from 29 to 39 was observed in the paternal X chromosome. Also, a rare recombination between DXS548 and FRAXAC1 was recorded in the maternal meiosis. Follow up of the neonate confirmed the same DNA genotype as in the CVS, but the child died of DiGeorge syndrome after four days and was subsequently found to carry a microdeletion of chromosome 22 using probe cEO. It is suggested that in this family the deletion of chromosome 22 is likely to be a chance event but the rare recombinant and the fragile X mutation might be causally related.

(J Med Genet 1995;32:236-239)

Fragile X syndrome is caused by a large expansion of a sequence of trinucleotide (CGG)

repeats in the first exon of the FMR-1 gene in Xq27.3.¹ Such large expansions do not arise de novo but are derived from "premutations", smaller expansions of the repeat which are clinically neutral but which show an increased tendency to expand further. In normal people the length of the repeat varies from 13 to 50 copies but is stably inherited through families, while repeat lengths over 60 show the progressively increasing instability characteristic of a premutation.² It is not known whether alleles between 50 and 60 are predisposed to mutate as they prove to be stable in some pedigrees and unstable in others; conceivably this may depend upon either the genetic background or the purity of the repeat sequence.³ Large expansions (>200 repeats) result in hypermethylation of the gene region and consequent failure of gene expression,⁴ giving rise to the clinical syndrome. In addition, the large expansion is associated with a fragile site inducible in cell culture, which gave the syndrome its name.

With the isolation of probes to the region⁵⁻⁷ and of PCR primers flanking the repeat,² direct diagnosis of carrier status and prenatal diagnosis by molecular techniques have now become routine. The PCR test permits rapid exclusion of fragile X in normal males and females upon detection of one, or two, normal sized fragments respectively. No new mutation sized allele has yet been reported, and until now no large scale expansions within the normal range have been observed although occasional increases by one or two repeats are documented.⁸ One example of a decrease in size from 29 to 21 repeats has recently been recorded in a Japanese family.⁹

We report here on a prenatal diagnosis for fragile X which found a remarkable combination of unusual events, including an expansion from 29 to 39 repeats on the paternal X chromosome, a rare recombination between FMR-1 and a close proximal marker on the maternal X chromosome, and a de novo sub-microscopic deletion in chromosome 22 which resulted in the death of the neonate at 4 days of age.

Materials and methods

FMR-1 ALLELES AND FLANKING MARKERS

Restriction enzyme digestion, Southern transfer, and hybridisation were carried out as described previously²⁵; PCR of the CGG repeat

Figure 1 Pedigree of the family showing the haplotypes (top to bottom) for microsatellite markers VK23, DXS548, FRAXAC1, the number of CGG repeats in FMR-1 (or, in ΔL subjects, the size of expansion (Δ) in base pairs), and FRAXAC2. Phase is inferred in most subjects assuming no recombination, but was proven in IV-1 by analysis of the father's haplotype and also that of the separated paternal X chromosome of IV-1 by somatic cell hybridisation (see Methods).

Figure 2 PCR analysis of the CGG repeats showing an expansion in the fetus IV-1 relative to the paternal (III-1) allele of 29 repeats. Size standards are fragments of pBR322MspI; PCR primers and conditions were as described by Fu *et al.*²

was as described by Fu *et al.*² Markers flanking FMR-1 were analysed using protocols described in reference 17 or in references therein.

SEPARATION OF THE X CHROMOSOMES

Hybrid cells were obtained by fusing fibroblasts from newborn umbilical cord with A9 mouse fibroblasts²⁶ and selection in HAT medium. The hybrids were screened to determine parental origin of the separated X chromosome by PCR analysis of alleles at the androgen receptor locus.²⁷

SEQUENCING OF THE CGG REPEATS

DNA was gel purified following PCR using flanking primers, and the purified fragments sequenced directly using the exonuclease deficient *Pfu* Cyclist sequencing kit (Stratagene) with modifications as described.²³ To detect the first base variation in the triplet array, only the G/T terminations are necessary when sequencing from the distal end. Approximately

50 ng of template was combined with 1 pmol of primer, the reactions denatured at 98°C for five minutes, and cycled through 30 elongation/termination steps of 98°C, 15 seconds and 70°C, one minute. Reactions were denatured at 95°C and run on 5% polyacrylamide sequencing gels. Autoradiography was carried out with Kodak XAR5 film at -70°C.

Results

The family pedigree is shown in fig 1. The proband (III-4), who was referred to us when his sister (III-2) became pregnant, is 34 years old and did not show an obvious fragile X clinical phenotype but was intellectually impaired. He was found to have 34% fra(X) cells and a large (Δ L) DNA expansion was detected by Southern analysis. Of the remaining family members, III-2 and II-3 also had Δ L expansions and II-2 had a premutation (Δ S). A chorionic villus sample was taken from IV-1 and DNA analysed by PCR and Southern blotting. No Δ L expansion was found in the CVS which had a normal female restriction fragment pattern using a double *Bst*ZI and *Eco*RI digest. However, PCR analysis (fig 2) showed an allele of 39 CGG repeats which was not present in either parent. Assuming the fetus's other allele of 30 repeats to be maternal in origin, the 39 repeat allele must either have arisen from an expansion of the 29 repeat allele of III-1, or from a different father.

To exclude the latter possibility, the CVS, together with III-1 and II-4 who also has a 39 repeat allele, were analysed with 25 microsatellite and VNTR markers on nine different chromosomes. The results (not shown) indicated convincingly that II-4 cannot be the true father of the fetus, while almost certainly confirming true paternity of the alleged father III-1. Thus III-1 is the only plausible source of the new 39 repeat allele which therefore represents a new rare expansion event within the normal range of repeat lengths.

Analysis of close flanking markers, together with the confirmation of III-1 as the father of the fetus showed another surprising anomaly (fig 1), that of a recombination on the maternal X chromosome between the marker loci FRAXAC1 and DXS548. By exclusion of the paternal alleles, the remaining maternal DXS548 and VK23 alleles in IV-1 were recombinant with respect to fra(X) status, while those of AC1 and AC2 were non-recombinant. DXS548 is approximately 140 kb from AC1, and until recently had been reported at zero recombination fraction within FRAXA.¹⁰ However, two recombinations between DXS548 and FRAXA have recently been seen in the same laboratory and it was suggested the map distance could be up to 6 cM.¹¹ Nevertheless, the absence of any previously reported recombinant between these two loci, the presence of linkage disequilibrium between DXS548 and the FRAXA locus, and their separation by only 140 kb makes a genetic distance greater than 1 cM implausible.

Having been assigned a low risk of fragile X, the pregnancy continued. An apparently

Figure 3 Sequencing of the FMR-1 array from III-1, III-2, and IV-1 (expanded X) using termination reactions designed to identify only the first base variation of each triplet. Reactions G and T indicate the presence of the triplet CCG and CCT, as read from the FMR-1 non-coding strand.

normal female infant weighing 2900 g was born at term. At the age of 3 days the baby suddenly became ill and was admitted to hospital with circulatory failure. A diagnosis of type B interrupted aortic arch, small aortic valve annulus, ventricular septal defect, and restrictive patent ductus arteriosus was made and a corrective operation was carried out the same day. Circulatory function was never fully regained postoperatively, and the baby died aged 4 days. Necropsy confirmed the congenital cardiac abnormalities and also showed a grade 3 intraventricular haemorrhage. No thymus or parathyroids could be identified on naked eye and histological examination of the anterior mediastinal contents.

These symptoms are typical of DiGeorge syndrome, and this diagnosis was confirmed by the detection of a submicroscopic del(22)(q11.2) using the cosmid probe cEO on in situ metaphase preparations, from skin fibroblasts. No such deletion was found in either parent, indicating a *de novo* mutation. We attempted to determine the parental origin of the 22q deletion without success: of four microsatellite markers from 22q11, two were non-deleted and two were uninformative. Analysis of the FMR-1 (CGG)_n sequence showed the same sized alleles (29 and 39 repeats) as had been detected in the CVS, on samples of cord blood, placenta, skin, and cultured skin and muscle fibroblasts. Two other trinucleotide repeat sequences were also analysed at the loci for myotonic dystrophy (DM) and Huntington's disease (HD), but no mutations in these repeats were recorded in any of the family (data not shown).

Separation of the paternal from the maternal X chromosomes in cell lines from cord fibroblasts was achieved by fusion with a mouse cell line, and subsequent DNA analysis confirmed that phase was as shown in fig 1. Sequencing of the trinucleotide tract was performed on the X chromosome of III-1, the normal X chromosome of III-2, and both X chromosomes of the newborn IV-1 (fig 3). This showed the following trinucleotide arrays:

Father III-1	(CGG) ₉ AGG(CGG) ₉ AGG(CGG) ₉	n = 29
Mother III-2	(CGG) ₁₀ AGG(CGG) ₉ AGG(CGG) ₉	n = 30
Daughter IV-1:	(CGG) ₉ AGG(CGG) ₉ AGG(CGG) ₉ X _m	n = 30
	(CGG) ₉ AGG(CGG) ₉ AGG(CGG) ₉ AGG(CGG) ₉ X _p	n = 39

As can be seen, the parental chromosomes differ only by one CGG repeat at the proximal end, each having two intercalating AGG triplets; the daughter's 39 repeat allele is related to the paternal 29 repeat allele by an extra (CGG)₉ + one AGG, but it is not possible to determine where in the trinucleotide array this insertion has occurred.

Discussion

It would seem that three rare events have occurred in a single generation of this family. There has been no reported association between fragile X and DiGeorge syndromes in the past but both are relatively common conditions and their association in this family may well be

the result of chance. However, while the two rare events in the same region of the X chromosome may also be independent, we think that they might well be related.

The recent evidence suggesting that expansion of the CGG repeat in FMR-1 might occur postconceptionally, rather than at meiosis,^{12,13} allows for a hypothesis about how the two events on the X chromosome may be explained. We suggest that the recombination in III-2 either (1) triggered or (2) was triggered by a locally acting instability gene (which could act both *in cis* and *in trans*) which, when passed on to the daughter, was responsible for the postconception expansion from 29 to 39 repeats on the paternally inherited X chromosome. It is difficult to test this hypothesis directly, but several lines of circumstantial evidence support the idea. (1) A family has been described in which a mutation from 42 to 43 repeats occurs on a paternally inherited X chromosome in one daughter who has also inherited a full fra(X) mutation from her mother, while the same 42 repeat allele remains stable in another daughter who inherited the normal maternal allele.¹⁴ This would be consistent with the idea of a maternally transmitted instability gene with postconception expansions, though the second unstable event in this case is only a single repeat difference. (2) In 121 female meioses analysed by Zhong *et al*,¹⁵ four new mutations at FRAXAC2 were observed, three of which were in a single sibship. By contrast, Richards and Mornet¹⁶ analysed 311 female meioses and found no example of an AC2 mutation, suggesting that one of the families of Zhong *et al*¹⁵ was unusually predisposed to mutation at FRAXAC2. (3) Expanded and thus unstable alleles of the FRAXA gene occur most frequently on rare or even unique haplotypes,¹⁷ suggesting that alterations of the haplotype, owing to recombination or mutation, in the vicinity of the FRAXA gene and its expansion are not independent events. (4) Instability in multiple, dispersed microsatellite sequences including triplet repeats has been shown in tumour tissue from colorectal cancers linked to mismatch repair genes on chromosomes 2 and 3¹⁸⁻²¹ which, although not necessarily by the same mechanism, supports in principle the idea of a gene promoting clusters of apparently heterogeneous DNA alterations.

The sequence data show that both parents' chromosomes have two intercalating AGG triplets at the same position and differ by only one CGG repeat at one end. The occurrence of interstitial AGGs is very common in FMR-1 and is held to confer stability on the repeat sequence, while those alleles with longer tracts of pure CGG are apparently more prone to slippage and expansion.^{14,22,23} Although no loss of an AGG has occurred in this case, the exact positioning of the AGG interruptions might prove to be critical and could indicate a potential for postzygotic interaction of maternal and paternal trinucleotide tracts leading to the unusual 10 repeat expansion. Sherman and Ashley²⁴ have proposed a model for FMR-1 expansion in which rare mutations of 10 repeats

occur in addition to the more common slippage by one or two repeats, and where each unit of 10 repeats mutates independently (a "branching" model). This study has provided a first documented case of a 10 repeat expansion from a normal allele, and also indirectly suggests a biological basis (in the positioning of intercalating AGG triplets) for possible independent mutation of 8, 9, or 10 repeat trinucleotide segments. Finally the observation of a Δ 10 repeat mutation on a common haplotype (7-3-4) for 548-AC1-AC2 lends support to our assertion¹⁷ that while one haplotype, 2-1-3, is associated with fra(X) cases derived from a single ancestral leap to a high end normal allele ("founder effect"), new mutations could still occur with significant frequency and with perhaps more rapid progress to a full mutation, on a range of other haplotypes.

Our thanks are extended to Nina James, Julia Fisher, and Hilary Bullman for helping with paternity testing; to Joyce Axelman and Melanie Dunn for helping with hybrid studies; and to Peter Scambler and the Wellcome Trust. Somatic cell hybrid studies were supported by NIH grant HD05465. We are also grateful to Evan Eichler for confirmation of the AGG interspersal pattern in DNA from IV-1 (paper in preparation).

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