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CASE REPORTS

Difficult diagnosis of the fragile X syndrome made possible by direct detection of DNA mutations

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Abstract

Genetic recombination near the fragile X locus (Xq27.3) has frequently been a problem in linkage studies of families in which the fragile X is segregating. This case report illustrates the resolution of a difficult situation in a fragile X family for whom cytogenetic studies were inconclusive and where recombination had twice confounded attempts at prenatal DNA diagnosis by RFLP analysis. Using a newly developed DNA probe, StB12.3, for direct detection of DNA instability in the fragile X locus, the presence of the fragile X was ascertained definitively in a prenatal DNA sample.

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The fragile X syndrome may be the most common cause of inherited mental retardation with an incidence of approximately 1 in 1500 males and 1 in 2500 females.[1] The mode of inheritance does not fit neatly into a mendelian pattern but may be considered to be X linked dominant with reduced penetrance in females. Carrier females may or may not be mentally retarded and may or may not show the fragile site cytogenetically. Transmission of the fragile X to offspring can occur through carrier females as well as normal carrier males, termed normal transmitting males, who can be shown to be obligate carriers of a fragile X chromosome but do not manifest any of the symptoms of the syndrome nor do they exhibit the fragile site cytogenetically.

Highly consistent and reproducible detection by cytogenetic studies of the fragile site in female carriers is difficult. The fragile X site can only be induced after cell culturing under conditions where the deoxypyrimidine pool is perturbed[2] and frequent false negative results occur. Failures to detect the fragile site cytogenetically in some patients, or detecting a small number of X chromosomes having questionable constrictions in Xq27.3, have led to many instances where attempts to diagnose the fragile X syndrome have been inconclusive.

During the 1980s it became possible to use restriction fragment length polymorphisms (RFLPs) flanking the fragile X locus to follow the segregation of the fragile X syndrome through a family with affected members. Occasionally, genetic recombination is a confounding problem when using RFLPs in linkage analysis. In some situations it is impossible to determine whether a recombination event has included the fragile X locus, leading to tremendous anxiety in the families being studied. We report here an example of the resolution of a difficult fragile X diagnostic situation using direct detection of mutations within the fragile X gene.

Case report

Our laboratory was contacted in December 1990, to perform prenatal DNA studies on amniocytes using RFLP analysis for a family where the uncle of the fetus had been diagnosed as having the fragile X syndrome. Fig 1 illustrates the family structure and the results of the linkage analysis. We had earlier performed a prenatal study in 1988 for a previous pregnancy in this family with the result that an unaffected male had been born (III.2). In the DNA study for the first pregnancy, the linkage analysis had shown that the mother (II.4) inherited a recombinant X chromosome from her mother (I.2). With the DNA markers in use in 1988 it was not possible to determine whether the recombination in patient II.4 had included the fragile X locus. Repeated cytogenetic studies of patient II.4 had all been negative for the presence of fragile X chromosomes. Thus, her carrier status had not been resolved when we were contacted about the second study. Using the DNA marker VK21 (*DXS296*), which was not available in 1988, we were able to estimate that she had approximately a 90% risk of being a fragile X carrier[3] based on the possibility of recombination occurring somewhere between the proximal marker cX55.7 and distally located VK21. Furthermore, the oldest child (III.1) of this woman had inherited her recombinant X chromosome and as a result had a high risk of being a fragile X carrier.

The fetus (III.3) in the present study was determined to be female by chromosome analysis and to have a single questionable cell, out of 300 studied, containing an X chromosome with a very slight constriction in the fragile X region. Linkage analysis showed that

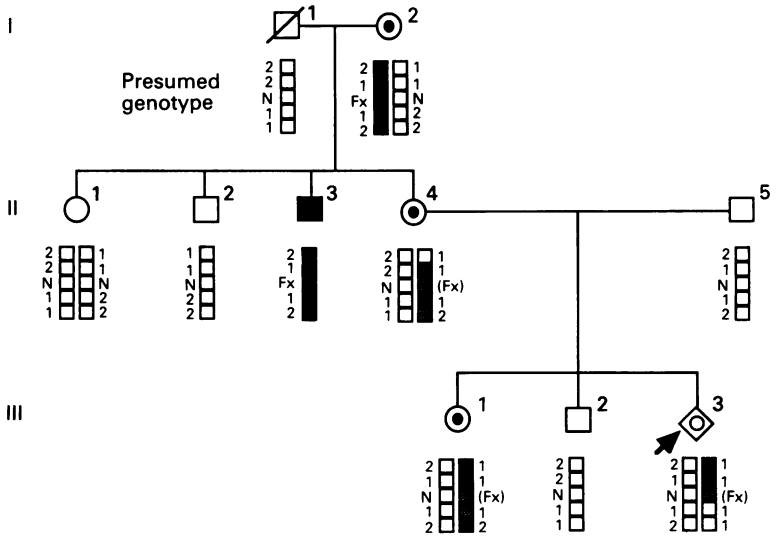

Figure 1 Linkage study of the family. Recombination events within the fragile X region have occurred twice in this family: (1) the X chromosome inherited by II.4 from her mother and (2) the X chromosome inherited by III.3. In both cases the recombination occurred between the informative DNA markers distal and proximal to the fragile X locus. II.3 is affected with the fragile X syndrome (black square). His mother, I.2, is cytogenetically negative but presumed to be a fragile X carrier before direct testing became available. The small connected boxes under each subject represent the X chromosome in the region near the fragile X locus and are used to display haplotypes constructed from linkage studies. Black boxes indicate that a chromosome segment was derived from an X chromosome bearing the fragile X mutation. White boxes indicate normal chromosome segments while grey boxes indicate segments whose derivation could not be determined because of uninformativeness or uncertain location of recombination endpoints. Thus, when II.4 was found to be a probable fragile X carrier, it became likely that III.1 had inherited the fragile X. The recombination in III.3 again confounded the risk analysis because the endpoints could not be defined. The order of RFLP loci found to be informative are: centromere-cX55.7 (DXS 105)-4D8 (DXS 98)-FRAX-VK21 (DXS 296)-St14 (DXS 52)-telomere.

the X chromosome inherited by the fetus from her mother (II.4) was a recombinant X chromosome. Since the closest DNA markers proximal to the fragile X locus were uninformative, the recombination endpoints could not be defined on the recombinant X chromosome. It was uncertain whether the fetus had a fragile X chromosome. As a result, the situation occurred where, for the second time in this family, DNA testing could not definitely determine the fragile X status of a female.

DNA STUDIES USING DIRECT DETECTION OF FRAGILE X MUTATIONS

Very recently it has become possible to detect mutations in the fragile X region directly and to perform diagnostic studies.[4] A newly identified gene (designated FMR-1) containing a highly repeated segment of the trinucleotide CCG appears to become unstable and susceptible to alterations in length in fragile X patients.[5-7] Fragile X carriers inherit a FMR-1 allele which has from 100 to 500 bp of extra DNA not found in the normal allele.[7] The additional DNA contains many copies of the CCG trinucleotide repeat and has been termed the 'premutation'. The region containing the CCG repeats has been termed 'mutable region'.[7] Full expression of the syndrome seems to be coupled to excessive methylation within FMR-1[7] and cytogenetic detection of the fragile X site has been speculated to be the large scale manifestation of the unstable segment.[5]

Southern blot analysis using a DNA probe (StB12.3)[7] located just distal to the CCG repeat segment, along with methylation sensitive (*Eag*I) and methylation insensitive (*Eco*RI) restriction enzyme digestion, can differentiate affected from normal males as well as normal transmitting males from normal males.[4] In addition, carrier females can be differentiated from normal females.[4] Restriction digestion with *Eco*RI will generate a 5·2 kb band from FMR-1 containing the mutable region. This DNA fragment may be further digested by *Eag*I into a 2·8 kb band, containing the mutable region, and a 2·4 kb band. Both the 5·2 kb and 2·8 kb bands can be detected by StB12.3. The *Eag*I site is located in a CpG island within or near to FMR-1 and is about 250 bp upstream of the mutable region. Normal males and females will have a 2·8 kb band on Southern blot analysis if the *Eag*I site is unmethylated, as is normally the case. Normal females also will have a 5·2 kb band generated from the inactive X chromosome where the *Eag*I site is blocked by normal methylation.

Using the direct detection approach we have been able to resolve the situation in our family which only recently seemed intractable. Fig 2 shows an autoradiograph produced from Southern blot analysis of the family. The various family members can definitively be categorised as to their particular fragile X status by this method.[4] Lane 1 contains DNA from the uncle of the fetus (II.2) and exhibits a normal male pattern of a single band at 2·8 kb. Patient II.3 (lane 2), the fragile X affected uncle of the fetus, has a smeared band of approximately 6 to 8 kb indicative of instability of the fragile X region. Amplification of the unstable region by an as yet unknown mechanism may explain the large expansion of the fragile X mutation. Lanes 3 to 5 contain DNA from the grandmother of the fetus (I.2), the mother of the fetus (II.4), and the sister of the fetus (III.1), respectively. The four band pattern seen best in lane 5 is typical of that found in fragile X non-expressing carrier females who have no manifestation of the fragile X phenotype or cytogenetic expression of the fragile site. All three have patterns indicative of non-expressing female carriers though it appears that with each succeeding generation some DNA amplification occurs at the fragile X locus in these females. Lane 6 contains DNA from the father of the fetus (II.5). Lane 7 contains DNA from the aunt of the fetus (II.1) and has two bands at 2·8 kb and 5·2 kb typical of a normal female. Lane 8 contains DNA from the normal brother (III.2) of the fetus. Fetal DNA is contained in lane 9. Overexposure of the autoradiograph was necessary to show a faint 5·2 kb band with the result that the background signal is increased.

Discussion

In the fetal DNA sample (fig 2, lane 9), the pattern of a smeared band at the 6 to 8 kb region plus a dark 2·8 kb band and faint 5·2 kb band may indicate two events: (1) amplification of DNA in the fragile X chromosomes

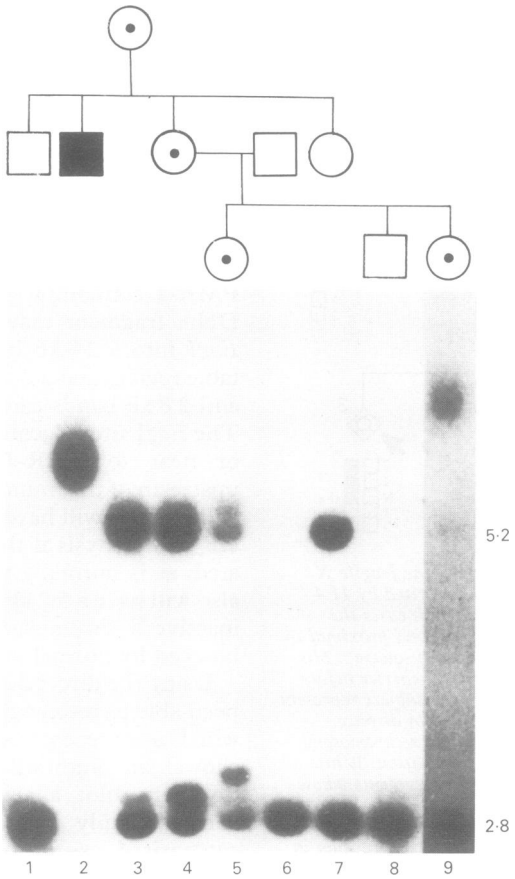

Figure 2 Direct detection of the fragile X mutation in the family. Each lane corresponds to the person indicated from the pedigree. See text for a discussion of each subject. A 0·8% agarose gel of 24 cm length was used to separate DNA fragments which migrate near the 2·8 kb and 5·2 kb fragments. It is important to use a relatively long agarose gel so that DNA fragments containing the amplified CCG region will separate from fragments containing the normal allele. Electrophoresis was performed overnight at 50 V then adjusted to 80 V for approximately four hours. The gel was stopped when the bromophenol blue (used as tracking dye) front reached approximately 2 cm from the bottom. The fidelity of restriction enzyme digestion was monitored using the DNA probe F33,[7] which detects 5·2 kb and 1·0 kb DNA fragments on EcoRI-EagI digests (not shown). It is important to stop the electrophoresis so that 1·0 kb fragments will not run off the bottom of the gel.

giving rise to the smear and (2) a skewed X inactivation pattern in the normal X chromosomes. The smeared band arises from heterogeneous DNA amplification of the mutant FMR-1 allele in somatic cells.[7] Though the molecular mechanism of alterations in the fragile X mutable region is not understood well at present, methylation of the amplified region presumably occurs to yield subpopulations of DNA fragments which migrate to the 6 kb to 8 kb region.

The presence of the very faint 5·2 kb band along with the much more intense 2·8 kb band suggests that X inactivation in this fetus has not occurred at random. The 2·8 kb and 5·2 kb bands should be present in relatively equal proportions if X inactivation were random. While there is evidence that the CpG island near FMR-1 shows little or no methylation on the inactive X in chorionic villi[4] (J-L Mandel, personal communication), the fetal DNA sample studied here was derived from cultured amniocytes where X inactivation presumably occurs at random. Since the *Eag*I site is contained within a CpG island which may regulate transcription of FMR-1,[8] the darker intensity of the 2·8 kb band indicates that the CpG island is unmethylated in the majority of cells and therefore more likely to produce FMR-1 transcription. This situation may have important repercussions if a threshold of FMR-1 expression has to be reached to allow normal cellular function, a hypothetical possibility at present. Deviation of X inactivation from the expected 50% of normal X chromosomes in a fragile X carrier female may lead to higher or lower levels of the FMR-1 gene product which potentially may affect phenotypic expression of the fragile X syndrome. However, the X inactivation pattern detected by studying amniocytes may not be representative of X inactivation in tissues responsible for the fragile X phenotype.

Currently, it is not possible to predict with a high degree of confidence whether the fetus will become mentally retarded or, if she is retarded, the degree to which she may be affected based on these results. The Southern blot pattern of carrier females who do not have expression of the fragile X syndrome is very frequently distinct from those carrier females who do have expression of the fragile X syndrome.[4] It appears that the four distinct band pattern typically seen in non-expressing carrier females correlates, to a high degree, with normal intelligence, no cytogenetic expression, and little or no expression of the dysmorphic features associated with the fragile X syndrome. In contrast, the pattern seen in the fetus studied here appears to be highly correlated with cytogenetic expression but less correlated with mental retardation. An initial estimate is that about 53% of fragile X females having a pattern similar to that seen in the fetal DNA studied here are mentally retarded.[4] Our laboratory has also found a comparable percentage of affected females with this pattern. Thus, the Southern blot patterns of carrier females have less predictive value of the potential of a female to be affected with the phenotypic features of the fragile X syndrome.

At nearly the same time that direct fragile X testing became possible, the baby was delivered. Cytogenetic testing performed on cells isolated from cord blood showed the presence of a low level of cytogenetic expression of the fragile site (3 to 4%). Currently, at 5 months of age, the baby has no overt signs of developmental delay but one might not expect to see any manifestation at this early time in her development. Thus, clarification of a possible correlation between the extent of DNA alteration as detected by direct DNA analysis and mental retardation in this child awaits the passage of time. The accumulation of more experience through testing of large numbers of females with a pattern similar to the fetus studied here should allow a better understanding of the relationship between the molecular alterations and mental retardation.

In summary, using a newly developed probe near the fragile X mutation, we have been able

to resolve an extremely difficult situation related to the segregation of the fragile X in this family. Clearly, this is one of the first of many examples where the power of the direct detection of the fragile X will be apparent.

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1 Nussbaum RL, Ledbetter DH. Fragile X syndrome: a unique mutation in Man. *Annu Rev Genet* 1986;**20**:109–45.
2 Sutherland GR, Baker E, Fratini A. Excess thymidine induces folate sensitive fragile sites. *Am J Med Genet* 1985;**22**:433–43.
3 Suthers GK, Mulley JC, Voelckel MA, *et al.* Genetic mapping of new DNA probes at Xq27 defines a strategy for DNA studies in the fragile X syndrome. *Am J Hum Genet* 1991;**48**:460–7.
4 Rousseau F, Heitz D, Biancalana V, *et al.* Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. *N Engl J Med* 1991;**325**:1673–81.
5 Verkerk AJMH, Pieretti M, Sutcliffe JS, *et al.* Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 1991;**65**:905–14.
6 Kremer EJ, Pritchard M, Lynch M, *et al.* Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence $p(CCG)_n$. *Science* 1991;**252**:1711–4.
7 Oberlé I, Rousseau F, Heitz D, *et al.* Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* 1991;**252**:1097–102.
8 Pieretti M, Zhang F, Fu YH, *et al.* Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* 1991;**66**:817–22.