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GENETICS IN CLINICAL PRACTICE

Guidelines for the diagnosis of fragile X syndrome

Ben A Oostra, Peter B Jacky, W Ted Brown, Francois Rousseau

Abstract

Direct DNA analysis of the fragile X mutation has become available with the isolation of DNA probes that detect the unstable DNA sequence containing the CGG repeat. We present the various alternatives of combinations of probes and enzymes that can be used for the diagnosis of fragile X syndrome. An overview is given of all the different available probes. A different protocol is presented for postnatal and prenatal diagnosis of fragile X syndrome. This includes Southern blot analysis as well as direct analysis of the CGG repeat by PCR amplification. We discuss the role of constitutional cytogenetic analysis in the diagnosis of mentally retarded subjects and cytogenetic analysis for the diagnosis of fragile X syndrome.

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Department of Cell Biology, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands.
B A Oostra

Department of Pathology/Cytogenetics, Kaiser-Permanente, Portland, Oregon, USA.
P B Jacky

Department of Human Genetics, New York State Institute for Basic Research, Staten Island, New York, USA.
W T Brown

Clinical Chemistry and Genetics, Hôpital St Francois D'Assise, Quebec, Canada.
F Rousseau

Correspondence to Dr Oostra.

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Fragile X syndrome is the most common inherited form of mental impairment, affecting approximately 1 in 1250 males.[1] The disease is associated with the expression of a folate sensitive site at Xq27.3, although the level of expression can vary from a few percent to as high as 70%. Recently, cloned DNA sequences which span the fragile site at Xq27.3 have been isolated.[2,3] At this site the FMR-1 gene has been identified[4] containing an unstable region of DNA which segregates with the fragile X phenotype.[2–6] The unstable DNA has been found to involve the repeat sequence $(CGG)_n$. There is a correlation between the length of the unstable sequence and the phenotype, thus permitting reliable and accurate diagnosis of fragile X carriers and patients by direct molecular analysis.[2–6]

Until quite recently, the diagnosis of fragile X syndrome has been based on the cytogenetic detection of a fragile site at Xq27.3. Numerous factors including specific tissue culture requirements, the type of cell population being studied, and the degree of affectedness and sex of an individual patient, contributed to the reliable cytogenetic detection of the fragile site and the diagnosis of the syndrome. An ad hoc committee was convened by P B Jacky at the Fourth International Workshop on the Fragile X Syndrome to recommend minimum cytogenetic standards for the preparation and analysis of the fragile X chromosome.[7] The guidelines described criteria for tissue culture methods for induction of the fragile site in vitro and for the analysis and interpretation of such chromosome preparations.

Direct DNA analysis of the mutations responsible for the clinical expression and transmission of the fragile X syndrome has begun to replace cytogenetic analysis. This transition reflects both the generally cheaper cost of establishing the diagnosis in subjects and through families with molecular methods, and the better reliability of DNA testing in establishing carrier status for the premutation in females and some males. The fragile X mutation is considered to be the result of elongation of a small DNA sequence containing a repeat of the trinucleotide CGG located in a 5'exon of the FMR-1 gene.[2–6,8] The increase of the number of repeats can be determined either by Southern blot analysis of genomic DNA or by PCR analysis of the CGG repeat. Two patients with a Martin-Bell phenotype have been described where (part of) the FMR-1 gene including the CGG repeat is deleted.[9,10]

During the Third International Fragile X Conference in Aspen, USA (1992), in the light of these developments, a protocol was developed and adopted to define the evolving relationship between cytogenetic and molecular testing for fragile X syndrome.

Cytogenetic diagnosis

We have entered a period of validation between conventional cytogenetic fragile X testing and molecular evaluation, and a number of reference laboratories have been encouraged to continue these studies in parallel for validation purposes, especially for prenatal diagnosis. It should be clearly acknowledged, however, that in the absence of specific fragile X cytogenetic testing, routine constitutional chromosome analysis will remain an integral part of the diagnostic workup of any patient, male or female, with mental retardation or significant developmental delay. These studies are appropriate on a person independent of a family history of intellectual handicap or obvious fragile X physical stigmata.[11] This recommendation reflects the occurrence of a large number of other constitutional type chromosome abnormalities that have been detected when patients were referred for fragile X analysis.

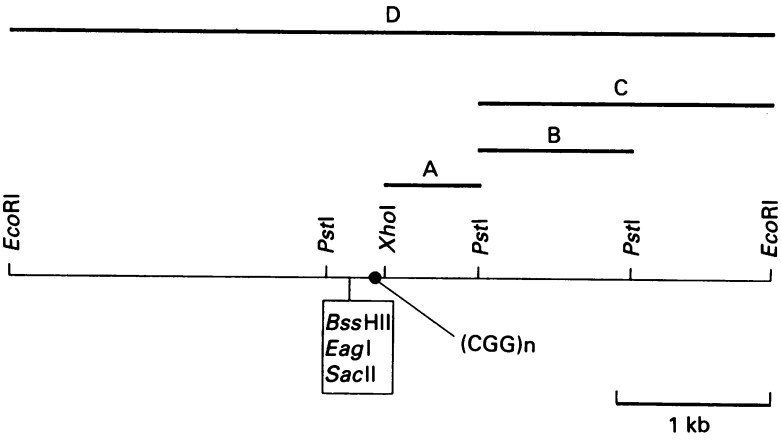
Fragile X chromosome studies under some

institutional protocols may remain the primary diagnostic tool in defining the initial proband in newly diagnosed families, to be followed then by a full molecular evaluation of the sibship. However, in general, DNA analysis will become the diagnostic tool to identify the proband in newly diagnosed families. Fragile X chromosome studies will in any case remain important in terms of understanding the significance of frequencies of fragile site expression and fragile site chromosome structure events as they relate to changes at the molecular level.

Recently, a new fragile site, *FRAXE*, was described in Xq28.[12] At the moment the only way to discriminate between *FRAXA* and *FRAXE* is by fluorescence in situ hybridisation using as markers cosmids located between *FRAXA* and *FRAXE*. The fragile site *FRAXE* is not associated with mental retardation.

Methods of DNA diagnosis

A CpG island described in Xq27.3 has been shown to be abnormally methylated in affected subjects.[13,14] Several probes can be used to detect this abnormal methylation[15] (figure). At the same time an increase in size of a fragment containing the CpG island and a CGG repeat can be detected. For example, in normal subjects a 5·2 kb *Eco*RI fragment is detected (table 1). The most prominent number of repeats in controls is 29.[8] In affected subjects an increase of this fragment is found with a length generally above 5·7 kb. In unaffected transmitting male carriers and a portion of female carriers a premutation allele between 5·3 and 5·7 kb is detected. Table 1 shows the different fragment sizes detected after digestion with different restriction enzymes. We propose for the detection of methylation of the CpG island the use of a double digest of *Eco*RI and the methylation sensitive enzyme *Eag*I. Using a probe distal to the CpG island (figure, probes B or C) the normal fragments are 2·8 kb in males and females and 5·2 kb on the female inactive X chromosome. The sizes of the *Eco*RI + *Eag*I fragments for the premutation and full mutation alleles are given in table 1.



Probes used in the diagnosis of fragile X syndrome. The restriction map of the 5·2 kb fragment containing the fragile site, the location of the CpG island (boxed), and the CGG repeat are given. The location and the names of the restriction fragments used to characterise the fragile X genotype are indicated.

Table 1 Fragment sizes detected with Southern blot analysis.

	Restriction fragment size (kb)					
	*Eco*RI*§	*Eco*RI + *Eag*I*§		*Bgl*II*	*Pst*I†	PCR‡
		M	F			
Normal	5·2	2·8	2·8+5·2	12	1·0	6–51
Premutation	5·3–5·7	2·9–3·3	2·9–3·3 5·3–5·7	NR	1·1–1·6	48–200
Full mutation	>5·7	>5·7	>5·7	>12·5	NR	>200

* 0·7% agarose gel using probes B or C (figure). † 1·0% agarose gel using probe A (figure). ‡ PAGE. § Partial *Eco*RI digest will generate a band 1·2 kb larger than the expected size(s). NR = not recommended.

Variation in the mutation in the FMR-1 gene can be detected after digestion of the DNA with different restriction enzymes. The choice of enzyme to use is dependent on the size of the insert. The detection of the small insert found in premutation alleles can be carried out with great accuracy using the enzyme *Pst*I. Full mutations are, on the other hand, often heterogeneous, resulting in a smear of bands. Mutations in fetal tissue are especially heterogeneous and we recommend performing a *Bgl*II digest.[6] The smear of bands will be compressed at the top of the gel with optimal detection of the full mutation.

As an alternative in molecular diagnosis, PCR analysis of the CGG repeat can be carried out. Two slightly different methods have been described by Fu *et al*[8] and by Pergolizzi *et al*.[16] The method of Fu *et al*[8] is applicable for the amplification of small alleles, but the amplification of larger (full size) alleles is so poor that they are undetectable. The PCR method described by Pergolizzi *et al*[16] uses a different amplification protocol and uses a hybridisation step with a CGG repeat probe to detect the amplification products.[16,17] Initial (unpublished) results suggest that, using either PCR protocol, the resolution by polyacrylamide gel electrophoresis of the shorter PCR products in the normal and premutation range is finer and allows for better size determination, whereas the use of agarose gel electrophoresis may allow for better visualisation of full mutations. The mosaicism observed in some subjects appears to be related primarily to the methylation status of the CpC island which is not detected by PCR. When a large premutation of a (small) full mutation is detected, in order to determine if mosaicism is present, complementary analysis by direct Southern blotting using digestion with a methylation sensitive enzyme such as *Eag*I is recommended. Further, when affected females with a normal allele size and a full mutation are suspected, selective amplification of the smaller allele may result in a single normal allele size and interpretation may be difficult. Such homozygous allele PCR results also should be checked by direct analysis.

Diagnostic protocols

We propose different protocols for prenatal and postnatal diagnosis (tables 2 and 3). Postnatally, a thorough diagnostic evaluation of a mentally retarded subject without a family history of *FRAXA* requires the molecular demonstration of the full mutation, or the

Table 2 Protocol for postnatal diagnosis of mentally retarded subjects and members of fragile X families.

Postnatal diagnosis	
Mental retardation	
Unknown aetiology	(1) Constitutional chromosome analysis (2) DNA *Eco*RI digest PCR P*
Family history (MR) without *FRAX* physical stagmata	(1) DNA *Eco*RI digest (2) Constitutional chromosome analysis (3) Optional *FRAX* chromosome analysis
Carrier testing (family history *FRAXA*)	DNA (a) *Eco*RI + *Eag*I digest (b) PCR F† or *Pst*I digest or PCR P*

* Pergolizzi *et al.*[16] Erster *et al.*[17] † Fu *et al.*[8]

presence of a chromosomal abnormality responsible for the intellectual handicap. The number of positive samples found in the DNA test and the number of cytogenetic abnormalities is of the same order, ±3% of tested mentally retarded subjects without a family history (unpublished results). Therefore, the method of choice is performing both tests on all samples. Fragile X cytogenetic testing under this circumstance may no longer be necessary. Subjects with a family history of mental retardation with or without *FRAXA* physical stigmata follow a similar diagnostic format but may under these circumstances include *FRAXA* cytogenetic studies for validation purposes. For routinely testing intellectually handicapped subjects the single digest is the method of choice. Use of the expensive double digest with a methylation sensitive enzyme in these cases is often wasted.[15] Frequently in *FRAXA* pedigrees diagnostic testing has to be extended to a large number of subjects, and must discriminate between normal alleles, premutation alleles, and full mutation alleles (table 1). There is not an exact cut off between large premutations and small full mutations (in the 200 repeat region). The presence of abnormal methylation allows the identification of the full mutations when the size is borderline. Special attention has to be given to the diagnosis of the premutation allele that has an amplification of between 48 and 200 CGG repeats. As is shown in table 1, a region of overlap is found between the smallest premutation alleles and the largest alleles found in normal subjects, approximately 50 to 60 repeats. Molecular evaluation of extended fragile X family members generally allows discrimination between normal and premutation alleles. In population screening it may be impossible to distinguish between normal and premutation alleles in this range.

A modification of this protocol is proposed for the prenatal diagnosis of fragile X syndrome (table 3). Full mutations can be detected after analysis of an *Eco*RI + *Eag*I digest, but it has to be noted that the methylation status in chorionic villi can be different from actual fetal tissue and the full mutation may be unmethylated.[18] The mutations in fetal tissue are very heterogeneous and we recommend performing a *Bgl*II digest also.[15] Premutation alleles can be detected by PCR or *Pst*I digest. As most subjects undergoing prenatal screening will have had an affected relative, a strong assay which can be carried out to discriminate between the two X chromosomes is the CA repeat assay. PCR analysis of flanking microsatellite CA repeats[19,20] can be carried out as a control and enables rapid detection of the normal genotype in more than half of at risk pregnancies.

Table 3 Protocol for prenatal testing of fragile X syndrome.

Prenatal diagnosis	
DNA*	*Eco*RI + *Eag*I digest or PCR* *Bgl*II digest PCR P* or F† or *Pst*I digest‡ As control: CA microsatellites (close to *FRAXA*)
Constitutional chromosome analysis§	

* Pergolizzi *et al*,[16] Erster *et al.*[17] † Fu *et al.*[8]
‡ In diagnosis of normal transmitting male in chorionic villi confirmation on amniotic cells or fetal blood is advised.
§ In a number of centres a cytogenetic test for fragile X will be carried out to validate prenatal DNA testing.

Table 4 Phenotype prediction after DNA analysis.

DNA test	Phenotype	
Normal	Normal	
Premutation	Carrier with normal phenotype	
Full mutation*	Male	High probability of MR
	Female	50–75% probability of MR 25–50% carrier with normal phenotype

* Including mosaics.

To date, the number of prenatal diagnoses analysing the number of CGG repeats is relatively small.[21–25] At the Fragile X Conference in Aspen it was proposed that a number of laboratories should continue to carry out cytogenetic testing of the fragile site as a validation of DNA testing in prenatal diagnosis. Data for the validation will be collected and presented during the next X Linked Mental Retardation meeting in 1993.

Molecular prenatal diagnosis is further confounded by other poorly understood factors. For instance, the mechanism of the extension from premutation to full mutation is still unknown. It is also not known when this expansion takes place. Therefore, one should be very careful in predicting whether a premutation found in chorionic villi accurately reflects the mutation pattern in fetal tissue. Until more data are available we recommend confirming the diagnosis of a premutation using CVS by subsequent analysis of amniotic fluid cells or fetal blood.

Reporting of DNA diagnostic results

The size of the CGG repeat and the presence of abnormal methylation appears to determine the phenotype. A normal phenotype is predicted for subjects with a premutation. Males with a full mutation (and abnormal methylation) always show the fragile X phenotype with mental retardation. Two patients with a Martin-Bell phenotype have been described where (part of) the FMR-1 gene including the CGG repeat is deleted.[9,10] The same phenotype is usually observed in males with a mosaic DNA pattern. Insufficient data are available to predict the phenotype of females with a full mutation. From the data available, the risk of showing mental retardation for females with a full mutation can be predicted to be between 50 to 75% (table 4).

Concluding remarks

We have presented strategies that can be applied for postnatal and prenatal diagnosis of fragile X syndrome. In the protocols we have combined different probe and enzyme combinations that have been presented in a number of papers.[2-6 8 16 17 21-24] The tests suggested here are sufficiently flexible to allow an attempt at standardisation, but are not the only way of carrying out the diagnosis. Using the guidelines, reliable postnatal and prenatal diagnosis of fragile X syndrome can be carried out. Special precaution has to be taken in diagnosing in CVS a premutation in a male fetus. Until more data are available confirmation of the diagnosis in amniotic fluid cells is recommended. A degree of standardisation in what is being assayed is needed for establishing risk estimates.

Direct DNA analysis of the fragile X mutation by molecular analysis has already proven its superiority for the diagnosis of the fragile X syndrome and is bringing to an end the era of uncertainty of fragile X diagnosis for patients and carriers.

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