DNA testing for fragile X syndrome in schools for learning difficulties

S F Slaney, A O M Wilkie, M C Hirst, R Charlton, M McKinley, J Pointon, Z Christodoulou, S M Huson, K E Davies

Abstract

Fragile X syndrome is the most common inherited cause of mental retardation. Early diagnosis is important not only for appropriate management of individuals but also to identify carriers who are unaware of their high risk of having an affected child. The disorder is associated with a cytogenetically visible fragile site (*FRAXA*) at Xq27.3, caused by amplification of a (CGG)_n repeat sequence within the gene at this locus designated *FMR1*.

Clinical and molecular studies have been undertaken to screen for fragile X syndrome in 154 children with moderate and severe learning difficulties of previously unknown origin. Southern blot analysis of peripheral blood showed the characteristic abnormally large (CGG)_n repeat sequence associated with fragile X syndrome in four of the 154 children. The findings were confirmed by cytogenetic observation of the fragile site and by further molecular studies. The families of the affected children were offered genetic counselling and DNA tests to determine their carrier status.

These findings show that there are still unrecognised cases of fragile X syndrome. Given the difficulty of making a clinical diagnosis and the implications for families when the diagnosis is missed, screening in high risk populations may be justified. The issues involved in screening all children in special schools for fragile X syndrome are discussed.

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Over the last decade, fragile X syndrome has been recognised as the second most common cause of mental retardation after Down's syndrome, affecting one in 1250 males and one in 2000 females.¹ The clinical features include generalised developmental delay of variable severity. Acquisition of speech and other cognitive skills is usually particularly affected² and there may be autistic features.³ Dysmorphism in fragile X syndrome has been described but the characteristic facial appearance, with a broad forehead and prominent ears, is not always obvious in affected boys and usually absent in girls. The face may become more distinctive with age and macro-orchidism tends to develop in postpubertal males, but by

then the most opportune time for making the diagnosis has been missed. The diagnosis of fragile X syndrome cannot be made exclusively on clinical grounds and depends on laboratory studies; however certain physical parameters may be useful in deciding who should be screened.⁴

The inheritance has an unusual X linked pattern.⁵ The majority of males inheriting the mutation are affected. However, in 20% the gene is non-penetrant, their intellect and physical appearance are normal but they are carriers of the disorder (normal transmitting males). Of females who inherit the mutation, 30% have some degree of mental impairment, but there are no specific dysmorphic features. For the 70% of carrier females who are intellectually normal there may be no outward sign at all of their risk of having mentally retarded children and it is frequently only after the birth of a second child with learning difficulties that a genetic cause is suspected.

The name of the syndrome originated from observation of apparent gaps or fragile sites at Xq27.3 in chromosomes derived from cultured lymphocytes in certain specific media.⁶ This observation has been used extensively as a diagnostic test for the syndrome in affected individuals, but in phenotypically normal carriers of both sexes the chromosomes almost invariably appear normal. Population screening for fragile X syndrome based on the cytogenetic marker, while successfully identifying new cases, has been limited by these problems.⁷

Fragile X syndrome is usually associated with an expansion in a gene called *FMR1* at Xq27.3 which contains an array of (CGG)_n repeats.⁸ In normal individuals the number of CGG repeats is less than 54, phenotypically and cytogenetically normal carriers have between 50 and 150 (so-called 'premutations') whereas affected individuals have larger expansions.⁹ The different insert sizes produce characteristic fragments after digestion with specific endonucleases which enables rapid and accurate assignment of individuals into each of these groups by DNA analysis.¹⁰

Recent advances in understanding the molecular basis of fragile X syndrome have made it possible to screen populations, especially for the intellectually normal carriers.¹¹ The aims of the present study were to (a) define a high risk population and screen for fragile X syndrome using DNA techniques, (b) estimate prevalence in the defined population, (c) assess the usefulness of clinical criteria in the screening programme, (d) provide further tests for

Department of
Medical Genetics,
Churchill Hospital,
Oxford OX3 7LJ
S F Slaney
S M Huson

Oxford Medical
Genetics Laboratories,
Churchill Hospital,
Oxford
R Charlton
M McKinley

MRC Molecular
Haematology Unit,
Institute of Molecular
Medicine, John
Radcliffe Hospital,
Oxford
A O M Wilkie

Molecular Genetics
Group, Institute of
Molecular Medicine,
John Radcliffe
Hospital, Oxford
M C Hirst
J Pointon
Z Christodoulou
K E Davies

Correspondence to:
Dr Slaney.

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the relatives of any new cases identified and assess the need for genetic counselling, and (e) evaluate the issues involved in introducing DNA screening for fragile X syndrome in high risk populations.

Subjects and methods

PATIENTS

The study population originated from a previously described, separate survey of children with unexplained learning difficulties, which looked for cases with the α thalassaemia mental retardation syndromes.^{12 13} The initial population of 567 children attended 11 schools for moderate and severe learning difficulties in Oxfordshire. The school and hospital notes were examined and excluded were children with a known cause for their learning difficulties (138, including eight with fragile X syndrome), or a previous normal full blood count (154). A letter was sent to parents of the remaining 275 children, requesting permission to perform a brief clinical examination and obtain a venous blood sample. This was granted in 164 (60%) of cases: blood samples were obtained from 159 and DNA made from 154 (103 males, 51 females, age range 3–18 years, including one pair of identical male twins). Clinical assessment at the time of venepuncture included measurement of head circumference and a photograph of the face. After the identification of the molecular basis of fragile X syndrome,⁷ ethics committee approval was obtained to test the DNA samples for the *FMR1* mutation (13 children had previously had a negative cytogenetic test for fragile X syndrome. Independently, the likelihood of the syndrome was assessed retrospectively using an additive clinical score (range 0–6) based on the presence of a head circumference >90th centile, ear prominence, particular speech or behaviour problems (one point each), and a family history of learning difficulties suggestive of X linkage (two points).

LABORATORY METHODS

DNA was extracted from whole blood and digested using the restriction endonuclease *Pst* I. The fragments were analysed by standard Southern blotting and hybridisation with the DNA probe Ox 0.55 as previously described.^{11 14 15} In addition, the females were screened with the *Bgl* II/Ox 1.9 combination, which served to compress any heterogeneous smears not detected using the initial procedure. Males in whom a normal *Pst* I/Ox 0.55 fragment was absent were reanalysed with probe Ox 1.9 after digestion with *Hind* III and *Hind* III/*Nru* I, enabling estimation of the size of heterogeneous expansions, and simultaneously assaying for abnormal methylation of the *FMR1* gene. Confirmatory DNA analysis and screening of at risk relatives was performed using the probe/enzyme combinations Ox 1.9/*Bgl* II, Ox 1.9/*Hind* III, and Ox 1.9/*Hind* III+*Nru* I.

Cytogenetic analysis for fragile sites was performed on 50 G banded cells per case.

*Results of laboratory studies in screen positive individuals from families I–IV. The number of each individual corresponds to the lane number in fig 1. Their clinical status is shown: affected boys (A), normal transmitting male (NTM), and carrier mothers (C). The hybridisation patterns and sizes of insertions in the *FMR1* gene in kilobases (kb) are indicated*

Family	Individual	Status	Size of DNA insertion in kb
I	4	C	0.23
	5	NTM	0.17
	7	A	Smear
II	8	C	Smear
III	9	C	1.12 and smear (mosaic)
	10	A	Smear
IV	11	C	0.23
	12	A	Smear

Lymphocyte cultures (72 hours) were established using 0.25 ml heparinised whole blood added to 5 ml RPMI 1640 medium supplemented with fetal calf serum (20%), penicillin (50 IU/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), heparin (1 IU/ml), HEPES (5 mM), phytohaemagglutinin (0.1 ml M form (Gibco)), and sodium bicarbonate (0.075%). Thymidine (200 mg/l) and colchicine (0.5 µg/ml) were added at 24 hours and one hour before harvest respectively. Harvesting was by standard procedures.¹⁶

GENETIC COUNSELLING

The parents of children in whom the results suggested the diagnosis of fragile X syndrome were informed by letter that a likely diagnosis for the mental retardation had been found and were offered genetic counselling. This included clinical reappraisal, explanation of the disorder and its inheritance, and blood sampling from the child and other family members who wished to be tested. Information and access to genetic services was also initiated for extended family members at risk.

Results

LABORATORY STUDIES

The screened population included 103 boys and 51 girls. In four out of 103 males, no normal fragments were present on the *Pst* I/Ox 0.55 screen. Further analysis showed, in each case, the presence of an expanded (CGG)_n array associated with adjacent methylation of the *FMR1* gene. All 51 females showed normal results using two different assays (see methods).

Repeat blood samples were obtained from three of the four boys, and all four of their mothers. Fragile X positive cells were identified in the two boys analysed cytogenetically. The results of molecular analyses are summarised in the table and fig 1. The affected boys all had large expansions confirming the results of the original screening test: these appear as smears on Southern analysis as they are unstable, tending to break down giving fragments of different sizes. All four mothers were heterozygous for insertions in the *FMR1* gene, confirming their carrier status. Two (mothers of cases I and IV) had small inserts in the premutation size range, the mothers of case II had a smear representing a methylated full mutation and the mother of case III had a

Figure 1 Southern blot analysis for fragile X syndrome in families I-IV. Hind III and Nru I double digested DNA was hybridised with the probe Ox 1.9. The hybridisation pattern obtained depends on the size of DNA fragments in each individual (table). Fragment sizes (indicated at the side in kilobases (kb)) were calculated by comparison with the Hind III marker (lanes 1 and 13). The position of normal fragments, 2.8 kb unmethylated and 5.0 kb methylated, are shown by the arrows. Affected boys showed a methylated smeared pattern of fragments (lanes 7, 10, and 12). Carrier females had either an unmethylated premutation (lanes 4 and 11), a smear representing a methylated full mutation (lane 8), or a mosaic pattern due to unmethylated and methylated fragments (lane 9). The normal transmitting male had an unmethylated premutation (lane 5), and the normal male and female had only normal bands (lanes 2 and 3), as did the normal brother in family I (lane 6).

mosaic pattern of methylated and unmethylated fragments.

CLINICAL FINDINGS AND GENETIC COUNSELLING

In each case, the pregnancy and perinatal history were normal and although three mothers reported concern about their child's development from the neonatal period, the age at referral for developmental delay varied from 3 months to 5 years. One child (II) had a previous normal chromosomal analysis at the age of 3 years: this was before the fragile site at Xq27.3 became a routine diagnostic test. The other three boys had received no genetic investigations. In only one child (case I) were the facial features suggestive of fragile X syndrome, but in all four boys the head circumference exceeded the 90th centile. Using the scoring system that took these and other factors into account, the four boys scored 4, 3, 2, and 1 point, out of a maximum of 6. The range of scores in the other 150 children was 0-4 (mode 0, mean 0.9). Thus all boys who were positive for fragile X syndrome scored above the mean, but overlapped with the scores in the other children.

The pedigrees of the four families are shown in fig 2. Additional mentally retarded individuals were identified in two of the families (III and IV) and there were many relatives at risk of being carriers. In the families of II and III, the maternal grandmother is presumed to be the

carrier in the top generation because the mother carries a full mutation (table); whereas in I and IV either the maternal grandmother or grandfather could have been the carrier (expansion of the (CGG)_n repeat from premutation to full mutation size occurs only in female meiosis^{9 10}). Follow up continues of other at-risk family members. To date, the two mentally normal brothers of case I have been tested: one has a normal *FMR1* gene, whereas the other is a premutation carrier (normal transmitting male). The latter individual will transmit the premutation to all his daughters (but none of his sons), whose own children will be at risk of inheriting the full mutation.

Discussion

In this study, a population of 154 children attending special schools, and with no established cause for their learning difficulties, was screened for fragile X syndrome using molecular techniques. Four new cases (3.9% of males, 2.6% overall) with the full *FMR1* mutation were detected. A further 275 children with unexplained learning difficulties were not screened, because either they had been excluded from the original α thalassaemia survey due to a previous normal full blood count, or no consent was obtained from parents, or DNA extraction was unsuccessful. Assuming these factors introduced no bias, a further 7.1 cases of fragile X syndrome would have been anticipated in this group. As only eight children with the syndrome out of 567 had been identified before this study, at least one third were undiagnosed. Given the anticipated number of 7.1 in the untested group, it is likely that over half the cases in the original population were undetected. We estimate the prevalence of fragile X syndrome in the special schools to be $(4+8+7.1)=19.1/567=3.4\%$. The childhood population in Oxfordshire was 78 811 at the time of testing, suggesting a minimum population prevalence of one in 4130.

Until recently, screening programmes for fragile X syndrome have relied on cytogenetic diagnosis. In a study of 347 children in special schools in Coventry, 29 were positive for the syndrome.¹⁷ In New South Wales 253 individuals positive for fragile X syndrome were found after 14 225 mentally retarded children and adults were screened.⁷ In New York cytogenetic analysis was carried out on a subset of 489 postpubertal mentally retarded males selected from a total of 1332 on the basis of 10 features of the syndrome; 43 were positive.¹⁸

The first published study of DNA based screening for fragile X syndrome was carried out on children with learning difficulties at schools in the Salisbury and Isle of Wight Health Authority areas.¹⁹ The investigators tested 180 boys and 74 girls out of an original population of 873 children with learning difficulties: four positive males (1.6% of those tested) were detected, giving an overall population prevalence of about one in 9000. In a second study, DNA analysis was used to

Figure 2 Pedigrees of families I-IV. The status of family members after DNA testing is shown.

screen blood samples from a population of 236 mentally retarded individuals (197 males and 39 females) referred to a clinical genetics department: 10 new males with the syndrome were found (4.2%).²⁰ A third pilot project, undertook DNA screening of saliva samples from 439 children (299 male, 140 female) referred for special education and detected one male and three females with full *FMR1* mutations (0.9%).²¹ The differing detection rates for fragile X syndrome of these three studies and the current study doubtless reflect the population screened, but all indicate the value of DNA based screening in identifying undiagnosed cases of fragile X syndrome. An attractive feature of the study by Hagerman *et al* was the use of buccal cells as the primary material for screening, avoiding the need for blood samples.²¹ Combining the four DNA based screening studies, only one individual carrying an *FMR1* expansion in the premutation size range (and presumably coincidental to the true cause of his learning difficulties) was identified. The frequency of premutations in the normal UK population is unknown, but a figure of one in 354 has been estimated in French Canadian women.²² It appears that coincidental detection of premutation carriers is unlikely to pose major additional difficulties

in counselling when screening mentally retarded individuals.

In comparing the efficacy of the cytogenetic and molecular DNA approaches to screening, DNA analysis is less labour intensive and more sensitive, specific, and cost effective than cytogenetic analysis.²³ However, one advantage of the cytogenetic test (with specific culture for fragile X syndrome) is the ability to detect a new fragile site, *FRAXE*, which lies distal to *FRAXA*.²⁴ Although fragility at *FRAXE* has been described in association with mental retardation in a few families with an atypical fragile X phenotype, it is not yet clear whether such cases are sufficiently numerous to warrant screening. The recent development of a DNA based test for *FRAXE*²⁵ will simplify further studies of its frequency in the mentally retarded population. Even if cytogenetic fragility tests were abandoned for screening, we would still advocate routine G banding cytogenetic analysis in all cases of unexplained learning difficulties, to exclude other chromosomal abnormalities.

In some cytogenetic screening studies, clinical criteria have been used to identify a subset of children with learning difficulties at particularly high risk of fragile X syndrome. A recent report suggested using a 13 item checklist combining physical and behavioural traits in screening mentally retarded males.⁴ We used a simpler approach comprising four of these items (presence of hyperactivity, perseverative speech, prominent ears, and positive family history) with increased head circumference, as relative macrocephaly may be the only suggestive feature in a prepubertal child with the syndrome. We found the most useful single clinical criterion was the head circumference, which exceeded the 90th centile in all four affected males but in only 33/150 unaffected children. Although these clinical criteria may be of help in enriching the screened population for cases of fragile X syndrome, no clinical scoring method is infallible: cytogenetic or DNA testing is essential to confirm or refute the diagnosis.

The introduction of a screening programme should follow standard recommended guidelines.²⁶ DNA testing for fragile X syndrome fulfils most criteria; as the most common inherited form of mental retardation it is an important health problem and the diagnosis in one individual often has implications for many relatives in the extended family. Treatment is not possible at the present time but accurate prenatal diagnosis and selective termination of affected fetuses can be offered,²⁷ which is the chosen option in some families.²⁸

A genetic screening programme has some special aspects for consideration. It has been recommended by the World Health Organisation that when a new genetic test becomes available it should first be assessed with the families of affected people.²⁹ A recent working party on screening for fragile X syndrome concluded that screening the general population is neither feasible nor ethical at present, and resources should be concentrated on diagnosing affected children and offering

services to their families including DNA testing, genetic counselling, and prenatal diagnosis.³⁰ As illustrated by this study, children at relatively high risk of fragile X syndrome may be ascertained with ease through special school registers; however, there is an increasing trend for children with mild learning difficulties to be educated in normal schools. An alternative means of ascertainment in the UK would be through the register of children who receive a statement of educational needs: the advantage of this is the inclusion of all children with learning difficulties regardless of severity.

In summary, this study illustrates that screening of schoolchildren with learning difficulties for the *FMR1* mutation is technically feasible, will identify previously undiagnosed index cases, and is acceptable to families. Further communication between parents, paediatricians, geneticists, teachers, and health economists is required to determine whether such screening should be offered on a nationwide basis.

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