Clinical, cytogenetic, and molecular analysis of three families with FRAXE

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Abstract

The probe StB12.3 has been used to screen the FMR-1 gene in 42 pedigrees with a distal Xq fragile site for expansion of the CCG repeat and aberrant methylation of the FRAXA locus. Four families did not have a FRAXA mutation and were investigated further. Fluorescent in situ hybridisation (FISH) and molecular analyses showed that three of these families had an expansion at FRAXE and one at FRAXF. Detailed psychiatric, psychological, and behavioural features of three families with FRAXE identified in the study are presented.

All the males who expressed FRAXE had a large methylated CCG repeat at FRAXE. All males with the mutation had some degree of mental handicap. This study illustrates the need for the FRAXE phenotype to be defined further.

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Fragile X syndrome, the most common form of inherited mental handicap, is associated with a chromosomal fragile site at Xq27.3.¹ Its unusual genetics and variable clinical course have made diagnosis and counselling difficult. New methods of diagnosis by examining the expansion mutation in FMR-1 are now used alongside more traditional methods of cytogenetically assessing the distal Xq fragile site.²⁻⁴ These developments have allowed more accurate identification of female carriers and normal transmitting males.

Some pedigrees without the expansion mutation at FRAXA have a distal fragile site, FRAXE.⁵ The clinical phenotype in pedigrees with fragility distal to FRAXA has not been shown to be related to the presence of the fragile site.^{6,7} A CCG expansion mutation in FRAXE has been identified and early evidence in this report suggests that the phenotype may correlate with the molecular change.⁸ A candidate gene for mild mental handicap at the FRAXE fragile site has recently been identified by Chakrabarti *et al.*⁹ A further fragile site FRAXF has been described in one family identified in this study¹⁰ and an expansion mutation has recently been reported in this condition also.^{11,12}

We have screened 42 pedigrees with a distal Xq fragile site known to South East Thames Regional Genetics Centre for the FRAXA mutation, which showed that in three families

the mutation was at FRAXE. Clinical, psychological, cytogenetic, and molecular analysis of these three families is presented.

Methods

PATIENTS

All families known to have a distal Xq fragile site through previous referral to South East Thames Regional Genetics Centre were contacted for inclusion. The following standardised instruments were used to evaluate psychiatric, psychological, and behavioural disturbance in three of the four families who did not have an expansion mutation in FMR-1: (1) British Ability Scales-short form¹³; (2) Childhood Behavior checklist, Parent Version¹⁴; (3) Parental account of Childhood Symptoms¹⁵; (4) Schedule of Handicaps, Behaviour and Skills.¹⁶

CHROMOSOMAL ANALYSIS

Two blood cultures were set up from each family member. For one, culture medium contained 300 µg ml⁻¹ thymidine as a fragile X inducer. The second culture in males used low concentration serum (5%) in medium TC199 and in females used fluorodeoxyuridine (4 × 10⁻⁷ mol/l) as a folate antagonist in standard lymphocyte cultures. Initially, 30 cells were examined from each of the two cultures and the overall background breakage recorded. A further 40 cells for males and 90 cells for females were scored from the culture with the highest background breakage. Positive results were recorded when fragile sites were shown in more than 4% of cells.

DNA ANALYSIS

Genomic DNA was initially extracted from leucocytes using a salt chloroform method,¹⁷ but because of recurrent difficulties with incomplete digestion this was later modified to a phenol-chloroform technique.¹⁸ The FRAXA mutation was analysed by digesting DNA (8 µg) with the restriction enzymes *EcoRI* and *EagI* (NEB/Promega). Fully digested DNA samples were electrophoresed, blotted, and hybridised with the radioactively labelled probe StB12.3⁴ as previously described.¹⁹ DNA from at least one normal male and one normal female control was included in every gel.

The FRAXE mutation was analysed by digestion with *HindIII* and *NotI* and the filters were probed with OxE 20.²⁰ Expansion mut-

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ations were sized by comparison of fragment mobility with a *Hind*III digest of lambda DNA end labelled with [α^{32} P] dCTP.

LOCATION OF THE FRAGILE SITES BY FISH

Fluorescent in situ hybridisation was carried out with cosmid probes 141R and VK21 labelled with biotin, previously described by Flynn *et al.*²¹ The X chromosome centromere was detected with the DNA probe DXZ1 (Oncor).

Results

FRAXA FAMILIES

The expansion and aberrant methylation changes were detected in 38 out of 42 pedigrees studied. There were no discrepancies between clinical phenotype, cytogenetic expression, and genotype.

FAMILIES WITHOUT THE FRAXA MUTATION

Four families were identified who had previously been diagnosed as affected with fragile X syndrome but who did not show the expansion mutation at FRAXA.

Family A

This is a sibship of three males who all show mild mental handicap and express a fragile site. Their mother (II.1) is illiterate and also expresses a fragile site. III.1 had an intelligence quotient of 54 with no significant verbal/performance discrepancy. Child behaviour profile indicated functioning in the top 2% of the population on obsessive-compulsive and hyperactive dimensions. Evaluation by the parents indicated mild concentration difficulties. Mild ritualistic/stereotypic tendencies were shown in a fascination with textures and a resistance to changes in routine.

III.2 had a developmental quotient of 50 generated using the Vineland scale of social development. Child behaviour profile showed functioning in the top 2% of the population on the aggression dimension. Teacher ratings confirmed marked classroom inattentiveness. Parental evaluation of attention showed mild concentration difficulties and restlessness with marked impulsiveness. Mildly repetitive speech was noted with ritualistic tendencies in the form of an insistence on routines.

III.3 had an intelligence quotient of 54 with no significant verbal/performance discrepancy. Child behaviour profile showed no abnormal score. Parental evaluation of attention indicated concentration difficulties and restlessness.

The head circumferences of the affected males were between the 50th and 75th centiles. Other family members examined include the maternal grandparents of the affected boys who were clinically and cytogenetically normal. The maternal grandmother was shown to carry the expansion.

Family B

This family has only a single affected male (II.1) aged 3.5 years available for study who expresses the fragile site. A developmental quotient of 65 was generated using the Vineland scale of social development. Child behaviour profile showed functioning in the extreme 2% of the population on multiple dimensions: social withdrawal, depression, immaturity, somatic complaints, schizoidness, and aggression. Parental evaluation of attention showed marked restlessness and fidgetiness and high activity levels. Social impairments were present in the form of gaze aversion, impaired interactive and symbolic play, and impaired imitation of social aspects of pretend play. Speech anomalies consisted of echolalia, repetitive speech, pronominal reversal, and muddling of sequences of words and phrases. Stereotyped repetitive bodily movements coexisted with a preoccupation with repetitive meaningless activities. There was a marked insistence on maintaining the constancy of the environment and routines. The above disabilities are sufficient to qualify for a diagnosis of typical autism.

His mother (I.1), who expresses a fragile site also, shows disturbances of speech and mood typical of females heterozygous for FRAXA.²² The affected boy and his mother are not dysmorphic and both have head circumferences greater than the 50th centile.

The proband in this family had a dead maternal uncle (I.4) who had both mental handicap and behavioural problems. A male first cousin of the proband (II.3) has similar clinical features but is unavailable for study.

Family C

This is a sibship of two brothers who express a fragile site. The family presented with III.1 aged 2.5 years who had language delay. He had small, simple pinnae and failure to thrive which was attributed to recurrent infections. He required ear reconstruction at 4 years which resulted in a left facial nerve palsy. He has attended mainstream school with additional classroom help, but is currently undergoing a statement of special educational needs aged 10 years. His intelligence quotient was 88 with no significant verbal/performance discrepancy.

Child behaviour profile showed functioning in the extreme 2% of the population on the following dimensions: obsessive-compulsive, hyperactive, and aggressive. Parental evaluation of attention indicated concentration difficulties with some impulsiveness, marked fidgetiness, and high activity levels. Some delayed echolalia and repetitive speech was present.

His younger brother (III.2) has been investigated for immunological deficiency because of recurrent infections and allergies. A deficiency of an IgG3 subclass was found. Fragile X status was examined because of concerns about his language development. He is currently in mainstream education with additional help, aged 6 years. Both boys have had middle ear disease and hearing loss which may have contributed to their learning disability. These brothers have head circumferences on the 50th centile.

Figure 1 Pedigrees A, B, and C. Shaded symbols represent subjects with mild mental handicap and hatched symbols represent subjects reported to be affected by family members. Percentages represent the percentage expression of FRAXE in lymphocyte cultures. Sizes of expansion detected at FRAXE are shown in base pairs (Southern blot estimations).

The mother of these boys (II.2) is clinically and cytogenetically normal, although she is shown to carry the expansion. Other relatives

who have been examined include the maternal grandmother and uncle. Both are clinically and cytogenetically normal, and without the FRAXE expansion.

IN SITU HYBRIDISATION STUDIES IN FAMILIES A, B, C, AND D
The location of in situ hybridisation signal from cosmid probes E2165 and VK21 confirmed that the fragile site in pedigrees A, B, and C is FRAXE. In pedigree D the fragile site was shown by in situ hybridisation to be FRAXF.¹⁰

MOLECULAR STUDIES FOR FRAXE EXPANSION IN FAMILIES A, B, AND C
The presence and size of the FRAXE expansion shown in family members available for testing is shown in fig 1. A sample blot with results from the families is shown in fig 2.

Discussion
This study has highlighted the occurrence of families diagnosed as having fragile X syndrome before the discovery of the expansion mutation at FRAXA, who have now been shown to have other distal Xq fragile sites with a different phenotype.
Three families with FRAXE are documented in this study. Sutherland and Baker⁵ identified FRAXE by in situ hybridisation, describing two families with a distal fragile site without the expansion at FRAXA; one of their families⁶ has subsequently been shown to have FRAXF rather than FRAXE.¹¹ In the two families which they describe the fragile site does not appear to be associated with a clinical phenotype, although in both cases the families were ascertained through a mentally handicapped proband.⁶ The females in these pedigrees have a high level of the fragile site expression in their blood in comparison to that seen in women who carry FRAXA.

Sutherland and Baker⁵ suggested that pedigrees reported by Oberlé *et al*,⁴ Nakahori *et al*,²³ and Rousseau *et al*²⁴ also have FRAXE. Two further families with FRAXE have been described with mild mental handicap in the probands without facial dysmorphism or macroorchidism.^{7,21} In these families the expression of the fragile site does not appear to be closely associated with the phenotype.
A report by Hamel *et al*²⁵ describes the clinical, psychometric, cytogenetic, and molecular data in a large FRAXE pedigree. Mild mental handicap was noted in all affected males and some females with the expansion. No specific phenotypic features were identified in this pedigree, as in those described here.
Mulley *et al*²⁶ reported six FRAXE pedigrees (including follow up of one of the original pedigrees reported by Sutherland and Baker⁵), with further evidence supporting a phenotype of mild mental handicap. One of their pedigrees was ascertained in an unbiased fashion after follow up of a family with FRAXA, and contains three subjects with mild mental impairment who have expansions ranging between 0.5 and

Figure 2 Southern blot analysis of families A, B, and C. DNA was digested with *HindIII* and *NotI* and after electrophoresis and subsequent blotting, the filters were hybridised with the probe OxE 20.

1.8 kb. In the others there were more handicapped people than would be expected by chance, although in general without other phenotypic abnormalities such as dysmorphic features or behavioural abnormalities.

The clinical features in males with expansions of more than 1 kb in the three pedigrees with FRAAXE reported here have mental impairment without dysmorphic features similar to that documented by Dennis *et al.*⁷ Hamel *et al.*²⁵ and Mulley *et al.*²⁶ The pedigree initially reported by Sutherland and Baker⁵ not to show a relationship between phenotype and genotype and later confirmed to have the FRAAXE expansion has been subsequently shown to have such a relationship. The families reported here provide confirmatory evidence of a phenotype of mild mental impairment without dysmorphism in FRAAXE. Behavioural problems seem rarely to be sufficient to cause social impairment in affected males, and may be no greater than those experienced by males with similar degree of learning disability from any cause.

We have identified 4/42 (9.5%) of pedigrees with a distal Xq fragile site as being without an expansion at FRAAXA, a similar proportion to that reported by other authors (table 1). Three out of four of the families in this study have FRAAXE and in these families there is some evidence for a clinical phenotype associated with the expansion mutation. There

Table 1 Proportion of families with a distal Xq fragile site and mental handicap studied who lack the expansion mutation of FMR-1

Reference	Total families studied	No without expansion	%
This study	42	4	9.5
4	53	4	7.5
22	68	5	7.9
27	43	5	11.6

is correlation between expression of FRAAXE and clinical features in eight subjects who all have expansions of 1 kb or more (fig 1). Two males can be considered secondary cases (family A, III.3 and family C, III.2); one is mentally handicapped (intelligence quotient 54), the other in mainstream education with additional help although without having formal assessment of his intelligence. Two females who are clinically normal and do not express the fragile site are shown to have expansions as well as two females with probable mild manifestations.

Comprehensive psychiatric, psychological, and behavioural evaluation of subjects from these pedigrees showed substantial behavioural heterogeneity. However, the striking observation is that the children showed minimal social impairment, apart from the proband in family B who was sufficiently disabled to fulfil diagnostic criteria for autism. This contrasts

with the majority of children who have FRAXA who tend to show a number of autistic-like social impairments in the presence of an otherwise friendly and sociable personality. They also show the cluttered litanic speech pattern which was not witnessed in the FRAXE children.²⁸ None of the four pedigrees without the expansion at FRAXA conforms to the clinical phenotype seen in fragile X syndrome when evaluated by formal psychological and behavioural assessments, although because of their developmental delay the initial diagnosis of fragile X syndrome had not been questioned.

DNA studies for FRAXA mutations are replacing chromosome tests as the diagnostic investigation of choice in some laboratories. Our results suggest that this should be undertaken with caution until there is more information about the clinical phenotype in FRAXE (and FRAXF), unless these expansions are also tested for.

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