

DNA Testing for Fragile X Syndrome in 255 Males from Special Schools in Singapore

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Abstract

Introduction: Fragile X syndrome, the most common cause of inherited mental retardation, results from unstable expansion of a trinucleotide (CGG)_n repeat in the FMR1 gene. Phenotypic expression is variable making clinical diagnosis difficult, while diagnosis by Southern blotting is relatively expensive and labour intensive. The prevalence in Singapore has not been studied. **Materials and Methods:** We developed a rapid screening test using a PCR analysis. We studied 255 males with unexplained cause for learning difficulties from 8 special schools. A clinical scoring system based on characteristic features described was devised. **Results:** PCR analysis showed absence of the band for the normal allele in 11 samples, 6 of which were confirmed by Southern blotting to be positive for FMR1 expansion, giving a 2% false-positive rate with PCR. Sensitivity of the PCR test was evaluated by performing Southern blotting in all PCR-normal samples; all of which were confirmed to be normal. This PCR test was shown to be highly reproducible. Clinical criteria were not predictive. **Conclusions:** Six (2.4%) new cases of fragile X syndrome were detected. There is a need to incorporate fragile X testing in routine screening of patients with developmental delay and learning difficulties. The use of PCR could eliminate the need for Southern blotting in up to 95% of cases. PCR analysis provides a simple, reliable and rapid tool for screening.

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Introduction

Fragile X syndrome is the most common inherited cause of mental retardation, affecting approximately 1 in 1250 males and 1 in 2500 females. It is associated with a cytogenetically visible fragile site in Xq27.3, termed FRAXA,^{1,2} which results from the unstable expansion of a trinucleotide (CGG)_n repeat sequence in the 5' untranslated region of the fragile X mental retardation gene (FMR1).³⁻⁶ Stable or normal alleles have 6 to approximately 54 repeats. Premutation alleles have approximately 55 to 230 repeats. Full mutations have more than 230 repeats. As a result of the repeat expansion, abnormal methylation of both a nearby CpG island and the repeat itself takes place, causing transcriptional silencing of FMR1.^{5,7-10} Lack of the protein product FMRP, an RNA-binding protein is responsible for the mental retardation.^{8,9,11} Methylation does not occur in

premutation alleles with normal transcription and normal FMR1 protein levels. Mosaicism is frequent in full mutations and can result in a mix of mutation and premutation alleles in different cells of the same individual. Methylation mosaicism can occur too, in which some cells are found to have methylation of FMR1 and some cells with no methylation of FMR1. Rare patients who have deletions of all or part of FMR1,¹²⁻¹⁴ or point mutations in FMR1¹⁵ have been reported.

Individuals who carry premutation alleles are unaffected. Premutations may expand to full mutations when transmitted by the mother and the risk varies according to the copy number of CGG repeats.^{16,17} Small variations in CGG repeat copy number may occur when transmitted through the paternal line, but full mutations are not produced.

The phenotype of fragile X syndrome includes a variable

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degree of mental retardation, prominent ears, long face, prominent jaw, high-arched palate, macrocephaly, macroorchidism and hyperactivity.¹⁸ Expression is variable and as affected individuals are fairly normal in appearance, fragile X syndrome is frequently undiagnosed.

The laboratory diagnosis of fragile X syndrome was largely by cytogenetic analysis of the fragile site,² which is however negative in normal transmitting males and female carriers with premutation alleles. This is now largely replaced by direct analysis of the CGG expansion mutation by Southern blotting.¹⁹ Using Southern blotting to screen routine referrals for fragile X syndrome, for which the great majority will be negative, is relatively expensive and labour intensive. More efficient methods using PCR analysis^{3,20} have been used for rapid screening.

The prevalence in Singapore is largely unknown. Few cases have been identified here and the diagnosis had depended on finding the characteristic phenotypic features described, and the expression of the fragile site on cytogenetic evaluation.

The aims of the present study were:

- (a) define a high-risk population in Singapore and screen for fragile X syndrome;
- (b) develop a protocol for rapid screening using a simple PCR analysis and using Southern blotting to confirm;
- (c) assess the usefulness of clinical criteria for screening.

Subjects and Method

The school records and available medical reports for 1900 children from 8 schools for special education in Singapore were screened. Three schools were for children with mild intellectual disability (IQ 50-70), while 5 were for children with moderate to severe intellectual disability (IQ <50). All children with known causes of mental retardation were excluded from the study (656 cases or 34.5%), while a letter was sent to parents of the remaining 1244 informing them of the study. Voluntary response for fragile X screening was 35.5% (442 cases), comprising 269 males and 173 females. The results for male subjects will be presented.

All interviews and clinical examinations were performed by the same doctor, with the following clinical features assessed: prominent ears, long face, prominent jaw, macrocephaly, high-arched or cleft palate, macroorchidism, simian crease, hyperactivity and preservative speech. A detailed family history of mental retardation and learning problems was elicited. A clinical scoring system was devised, with 1 point for each of the above clinical features and 2 points for family history suggestive of X-linked mental retardation. Venous blood was sampled from 255 male subjects, with 14 excluded from the study because a

known diagnosis was established from clinical assessment or venepuncture refused.

Laboratory Method

PCR amplification of the FMR1 repeats

DNA was extracted from whole blood using the standard protocol of lysis, proteinase K digestion, followed by extraction with phenol and chloroform.²¹ Amplifications were carried out in a thermocycler in 50 µl reactions containing approximately 300 ng of DNA template, buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl and 1.5 mmol/l MgCl₂), 10 pmol each of primers g (forward primer: 5'AGTGCGACCTGTCACCGCCCTTC3') and f (reverse primer: 5'AGCCCC-GCACTTCCACC ACCAGTCTCTCCA3'),³ 1 U of AmpliTaq polymerase (Perkin Elmer), 10% dimethyl sulphoxide (DMSO), and 200 µmol/l each of dATP, dCTP and dTTP, and 100 µmol/l each of dGTP and 7-deaza-2'dGTP. The reactions were denatured at 95°C for 8 minutes followed initially by 5 cycles of denaturation at 95°C for 90 seconds, annealing at 70°C to 66°C (decreasing by 1°C per cycle) for 60 seconds, and elongation at 72°C for 120 seconds ("touch-down" procedure). The samples were then subjected to 35 cycles of amplification at 95°C for 90 seconds, 65°C for 60 seconds, 72°C for 120 seconds; with a final extension of 10 minutes at 72°C. A total of 25 µl of product was analysed by electrophoresis in a 1.5% agarose gel containing ethidium bromide.

The presence of a band in the correct size range (approximately 522 bp) for the normal allele excludes the presence of an expansion mutation at the FRAXA locus (Fig. 1). We had used a biotinylated (CGG)_n oligonucleotide to hybridise the PCR product to confirm the specificity of the amplification.

FMR1 analysis by Southern blotting

Genomic DNA was double digested using the restriction enzymes EcoR1 and Eag1.¹⁹ The fragments were separated electrophoretically, transferred to a positively charged nylon membrane and hybridised using a digoxigenin-labelled DNA probe. The DNA probe used was generated by PCR amplification of a 451 bp region immediately downstream of the CGG repeat in FMR1 gene using a pair of primers fmr9U.1 and fmr10L.2¹⁰ in the presence of digoxigenin-dUTP. Hybridisation was carried out in Easi-Hyb (Boehringer Mannheim) at 42°C overnight, with the probe concentration of 4 to 5 ng/ml. Stringency wash was carried out at 65°C, with a final wash of 0.1X SSC, 0.1% SDS for 30 minutes. Chemiluminescent detection was performed using CDP-Star (Boehringer-Mannheim). Autoradiography for an initial exposure of 15 minutes was used to assess the intensity of the signal, followed by an

adjusted exposure of 30 to 90 minutes.

Normal females will show two bands, one at 2.8 Kb from the active X and one at 5.2 Kb from the inactive X that is methylated (produced by lyonisation). An unaffected male, having only a single active X will produce only the 2.8 Kb band. A premutation appears as a small increase in size of the 2.8 Kb band or as an additional band just above the 5.2 Kb band. Males with the full mutation will produce a diffuse band or a smear of greater than 5.2 Kb in size (Fig. 2).

Results

A total of 269 males were seen in the clinic. Fourteen were excluded from the study because they had a known cause for learning difficulties ascertained from history and clinical examination, or because venepuncture was refused.

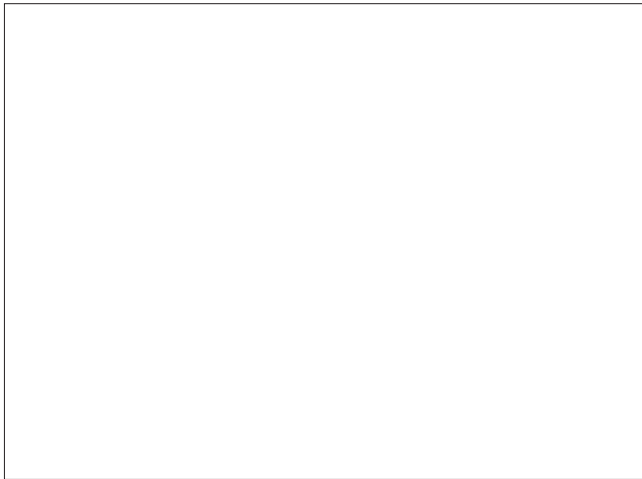


Fig. 1. PCR amplification products from the FRAXA locus analysed on 1.5% agarose gel. Lanes 1, 3-7, 9 normal males; lanes 2 and 8 are males with FRAXA expansion.

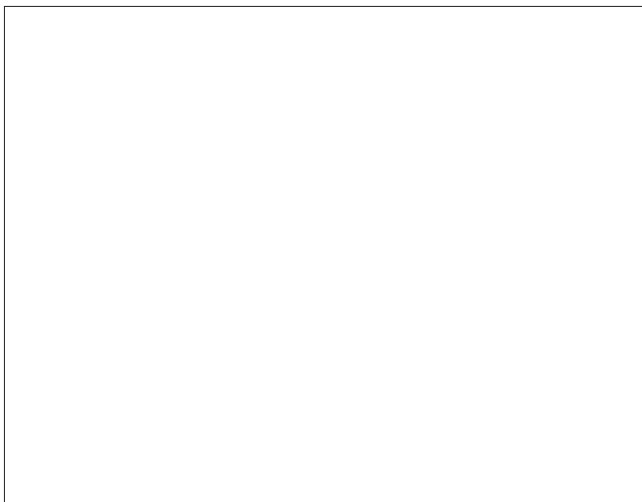


Fig. 2. Southern analysis for detection of FMR1 mutation in DNA digested by EcoR1 and Eag1. Lanes 1 (control) and 4 (mother of affected) show premutation bands; lanes 5 to 10 show single 2.8 Kb band of normal males; lanes 2, 3, 11, 12 show smears above 5.2 Kb from fragile X syndrome patients with full mutation.

The 255 males studied included three pairs of brothers and one pair of first cousins. One hundred and twenty-two (47.8%) subjects were from schools for those with moderate to severe intellectual disability, the other 133 (52.2%) were from schools for those with mild intellectual disability. Their ages ranged from 5.3 to 18.8 years, with a mean of 12.1 years. There were 163 (63.9%) Chinese, 65 (25.5%) Malays and 27 (10.6%) Indians.

The majority of subjects presented with a history of developmental delay between the ages of 1.5 to 3 years (189 or 74.1%). Eight had previous diagnosis of autism, 28 had history of epilepsy and 15 had history of hyperactivity. All had been previously assessed by a medical practitioner and most had IQ assessments as a pre-admission requisite to the special schools. There was a family history suggestive of X-linked mental retardation in 3 boys, including a pair of cousins. Twenty-six others had a non-specific family history of mental retardation. Prominent ears were seen in 72 (28.2%) boys, long face in 18, high-arched palate in 37 and cleft palate in 3. Simian crease was seen in 17 boys. None had macroorchidism (Table I).

PCR analysis showed no product in 11 samples. Southern blotting showed 6 of the 11 samples had full mutation, giving a false-positive rate of 2% using PCR analysis. Two of the affected boys were a pair of first cousins. To assess sensitivity of the PCR technique, Southern blotting was performed on all the samples that had normal PCR product. All were shown to be absent for the FMR1 expansion on Southern blotting. We found the PCR method to be highly reproducible, showing repeatedly no PCR product in samples with confirmed full mutation subjected to 15 PCR tests.

The 6 boys who were positive for FMR1 expansion showed on Southern blot smears of 400 to 1400 repeats. Their ages ranged from 5.6 years to 15.5 years (5.6, 8.9, 10.2, 10, 12.6, 15.5 years). The mean age at diagnosis was 10.5 years. One boy was assessed previously at admission to the special school to have an IQ score between 50 to 70, while the other 5 had IQ scores of less than 50. All had 2 or more of the physical features (2, 2, 3, 5, 5, 5). The range of scores in the other 249 boys was 0 to 4 (mode 0, mean 0.93) (Table II). The 6 affected boys all presented with history of developmental delay between the ages of 2 to 3 years. A history of epilepsy was present in one subject, hyperactivity in another, while none had history of autism. Three had family history suggestive of X-linked mental retardation including the 2 first cousins. A common feature seen in all 6 affected boys was the presence of prominent ears. A long face was present in 3, macrocephaly in 2. The 2 cousins both had cleft palate. One other boy had a high-arched palate. None had macroorchidism; the 15.5-year-old boy was noted to have testicular volume of about 25 ml.

TABLE I: CLINICAL FEATURES ASSESSED

Clinical feature	FRAXA positive males (n = 6)	FRAXA negative males (n = 249)	P value (Fisher's exact test)
Prominent ears	6 (100%)	66 (26.5%)	0.00043
Long face	3 (50%)	15 (6.0%)	0.00521
Macrocephaly	2 (33.3%)	37 (14.9%)	0.22932
Prominent jaw	0 (0%)	0 (0%)	-
High-arched/cleft palate	3 (50%)	37 (14.9%)	0.05109
Simian crease	1 (16.6%)	16 (6.4%)	0.34178
Macroorchidism	0 (0%)	0 (0%)	-
Hyperactivity	1 (16.6%)	14 (5.6%)	0.30752
Preservative speech	0 (0%)	0 (0%)	-
F/H X-linked MR	3 (50%)	0 (0%)	0.00001

TABLE II: CLINICAL SCORE

Clinical score	FRAXA positive males (n = 6)	FRAXA negative males (n = 249)	Total
0	0 (0%)	142 (57.0%)	142
1	0 (0%)	68 (27.3%)	68
2	2 (33.3%)	23 (10.8%)	25
3	1 (16.6%)	13 (5.2%)	14
4	0 (0%)	3 (1.2%)	3
5	3 (50%)	0 (0%)	3
>5	0 (0%)	0 (0%)	0

Using the Mann-Whitney U test, the difference in the distribution was found to be statistically significant

Genetic counselling and further testing were offered to all families of the affected. This was accepted by the families and Southern blotting was used for assessment. Family studies confirmed the presence of premutation in the mothers of five of the boys and a full mutation in the mother of the sixth boy. The maternal grandmother of the sixth boy was shown to carry a premutation. The extended family of the two affected cousins was screened and three other cousins were confirmed to have the full mutation

(Fig. 3). One mother gave a history of premature menopause, while another had a history of irregular menstruation, features reported to be frequently seen in females with premutation.²² Cytogenetic analysis performed in 3 affected boys was positive for the fragile site.

Discussion

In this study, we have screened a population of 255 males from the special schools who have no known cause for their learning difficulties. The voluntary response rate seen (35.3%) is much lower than that of similar studies conducted in Western populations. A possible reason is perhaps that there is still a social stigma attached to having a child with learning difficulties in our society. Parents may be unwilling to subject uncooperative children to venepuncture. Parents may also find it inconvenient and unnecessary to bring their otherwise well children to the clinic. The lack of a positive family history may also lead to the erroneous belief that there is no genetic cause. We also noted that the response rate from children from the schools for those with more severe intellectual disability (30.5%) was lower than that from the other group with less severe intellectual disability (41.0%).

We have evaluated a PCR analysis to screen for presence of the normal allele. Southern blotting was then used to confirm presence of the FMR1 expansion in those with no PCR product. Six new cases (2.4%) with the full mutation have been detected. This is comparable to results of other studies on high-risk populations, where detection rates of 2% to 5% have been reported.²³⁻²⁵

Clinical scoring was found to be generally not predictive for diagnosis. The most common feature among the 6 affected boys was presence of prominent ears, which was also observed in more than a quarter of the study population. A family history suggestive of X-linked mental retardation appeared to be the strongest predictive factor in this study.

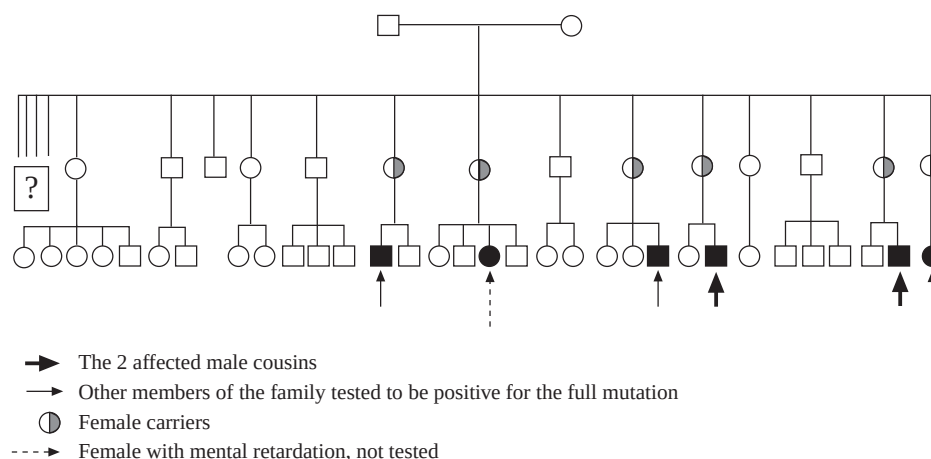


Fig. 3. Family pedigree of 2 cousins.

The clinical characteristics are largely subjective signs and not specific to the condition. Though our findings suggest that boys with fragile X syndrome were generally more likely to have a higher clinical score, there was still an overlap when compared to those who tested negative.

Molecular DNA analysis has been found to be less labour intensive and more sensitive, specific and cost-effective compared to cytogenetic analysis.²⁶ However, one advantage of cytogenetic analysis is the ability to detect the other fragile site, FRAXE, which is also associated with mental retardation. Routine G banding cytogenetic testing should still be performed in cases of unexplained learning difficulties to exclude other chromosomal abnormalities.

Southern blotting, though established for diagnosis of fragile X syndrome, is however, a relatively expensive and labour intensive procedure especially when used in the context of screening routine referrals where the majority of cases will be negative. PCR analysis allows for more rapid and simple screening.

PCR amplification of CGG repeats in the FRAXA fragment has presented difficulties due to the high G+C content of the region. The incorporation of 7-deaza-2'dGTP to stabilise secondary DNA structure allows successful amplification of the CG rich region.²⁷ PCR products with a high content of 7-deaza-2'dGTP however, do not stain efficiently with ethidium bromide.²⁸ As a result, most current methods involve radioisotope labelling or blotting and detection with biotinylated or chemically modified nucleotides. We have used in our PCR analysis 2 primers, which had sequences previously described³ but which have not been described to be used together in a PCR analysis for screening of the normal allele. We found that a 1:1 ratio of 7-deaza-2'dGTP:dGTP in our reaction resulted in successful amplification of FRAXA allele within the normal range and efficient staining of the PCR product with ethidium bromide, allowing for direct visualisation under ultraviolet light, thus eliminating the need for radioactive tests. A false-negative result would be obtained in an affected male who was a mosaic for a normal allele and a full mutation. However, no such cases have been reported to date. The usual precautions for the prevention of contamination of the PCR product must be observed.

Using this PCR analysis could eliminate the need for Southern blot in up to 95% of routine cases.

This study shows that there is a significant number of males with fragile X syndrome in the high-risk population in Singapore. Due to the lack of specific characteristic features, the lack of easily available testing and lack of understanding of the condition, these cases have previously remained undiagnosed, and there are still probably more unrecognised cases.

As the most common inherited form of mental retardation, fragile X syndrome is an important health problem and the diagnosis in one individual often has implications for many relatives in the extended family. There is a need to incorporate fragile X testing in routine screening for developmental delay and learning difficulties. Greater understanding of the condition should also be provided to healthcare and educational professionals to facilitate early identification and referral. Although no known cure is currently available, educational and therapeutic interventions may be of benefit.^{29,30} Medical treatment can be given for associated attention deficits and hyperactivity.³¹ Screening for carrier status in families of the affected child provides information on the risk in future pregnancies and allows families to make informed decisions about family planning and prenatal screening.

In conclusion, we have developed a PCR analysis that is a simple, reliable and rapid tool for initial screening of fragile X syndrome in males. Southern blot analysis should still be used for confirmation of the presence of an expanded allele. Clinical features have been found to be generally not predictive and routine screening should be considered in high-risk population even when the characteristic phenotypic features described are not present.

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