Fragile X syndrome in Calcutta, India

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SUMMARY. Fragile-X-linked mental retardation usually results from amplification of the CGG repeat in the 5′ untranslated region of the *FMR1* gene. To assess the extent of variation of the CGG repeat in the population from the eastern region of India we studied 98 mentally retarded individuals living in and around Calcutta and identified 21 distinct alleles ranging in size from 8 to 44 CGG repeats. A repeat size of 28 was the most frequent; this value is different from the most frequent repeat size found in other studies, indicating a racial or ethnic variation. Patients with the clinical features of the syndrome have been found to carry expanded CGG repeats. Thus, it can be inferred that the expansion of CGG repeats may be a frequent cause of the syndrome in our population.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation, with an incidence of 1:4000 in men[1] and 1:2000–2500 in women.[2] Almost all cases of the condition are caused by the expansion of the $(CGG)_n$ trinucleotide repeat in the 5′ untranslated region of the *FMR1* (fragile X mental retardation) gene.[3–8] The repeat is polymorphic: in the non-retarded population it varies from 6 to 54 and the most frequent allele observed in the Western population has 29 CGG repeats.[9] Individuals having 55–200 repeats are the carriers of premutations, whereas those having more than 200 to several thousands repeats are said to bear full mutations. Individuals with full mutations are those clinically affected.[9] Both the mutations are meiotically unstable and mosaicism in individuals suggests mitotic instability as well. Expansion of premutations to full mutations occurs only in female meiotic transmissions.[7,9]

The *FMR1* gene has been localized to the fragile site Xq27.3.[7] The fragile site appears as a non-staining gap of variable width under appropriate *in vitro* conditions. At the molecular level it has been identified as a tandemly arranged repetitive sequence of CGG trinucleotides included in the 5′ untranslated exon of the *FMR1* gene immediately adjacent to or embedded within a CpG island. Expansion of the triplet repeat is often accompanied by hypermethylation of this CpG island when the number of repeats increases to ~230, causing transcription of the *FMR1* gene to shut off. The CpG island is also methylated on the inactive X chromosome of all women. There is an *Eag*I restriction site at this CpG island which is lost upon hypermethylation. This loss of the *Eag*I site has been used to assess the methylation status of the CpG island.

In the present study, the variation of the CGG repeat was examined among individuals living in and around the city of Calcutta, a part of eastern India. Along with the study of CGG polymorphism in normal individuals, an attempt has been made to observe the frequencies of premutations and full mutations in the population.

MATERIALS AND METHODS

Subjects

DNA analyses of the CGG repeats were carried out on samples from 158 individuals (97 men, 61 women). Of these, 60 were samples collected from healthy volunteers (students and research

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scholars of the department). The exclusion criterion was the absence of CGG expansion. The distribution of CGG repeat sizes in this group closely resembled that of control samples used in previous studies.[9] The other 98 samples were collected from subjects who showed unclassified mental retardation and were either attending institutions for the mentally handicapped or were referred to us from various neurological clinics in Calcutta. Consent was obtained from the subjects or their guardians.

Clinical assessments of the patients

The two boys, F36 and F41, in family A (*see* Fig. 1) were aged between 16 and 18 years and had severe problems suggestive of the syndrome. They had large head circumference, lop ears and macro-orchidism. Behavioural peculiarities included inattentiveness and hyperactivity. The maternal uncle of these two boys, F37, had an elongated face with large ears and gaze aversion. His IQ was around 60. Individual F41 had an 11-year-old brother, F40, who was severely retarded and was found to carry a full mutation. F38 and F39, the mothers of probands F36 and F41, respectively, were found to be leading normal lives. Another patient, F45, was a 60-year-old man with elongated facies, large ears, macro-orchidism and an IQ of around 30. F23 was a female patient with developmental delay and psychiatric abnormalities.

Polymerase chain reaction analysis

DNA was extracted from fresh leucocytes using the standard protocol of Miller *et al.*[10] Polymerase chain reaction (PCR) was carried out as previously described[9] using 100 ng of genomic DNA, 3 pmol each of primer C and 571R, 3–5 μCi of [^{32}P]dCTP and 1·75 U of enzyme mix from the Expand™ Long Template PCR system (Roche Molecular Biochemicals, Indianapolis IN, USA) in a final volume of 15 μL. The final concentrations of the other constituents were: dATP, dCTP, dTTP, 200 μmol/L; dGTP, 150 μmol/L; 7-deaza-dGTP, 50 μmol/L; dimethylsulphoxide, 100 mL/L; Tris–HCl pH 9·2, 50 mmol/L; $(NH_4)_2SO_4$, 160 mmol/L; $MgCl_2$, 17·5 mmol/L. After denaturation at 95°C for 10 min, the enzyme was added and amplification was carried out for 30 cycles (95°C, 1 min; 65°C, 1 min; 68°C, 2 min). Radioactive PCR products were loaded onto a denaturing 6% polyacrylamide gel with 95% formamide loading buffer and alleles were identified by sizing relative to allele specific markers of known CGG repeats. All PCRs were performed on the Biorad Gene Cycler and all the primers used were procured from Life Technologies (Grand Island NY, USA).

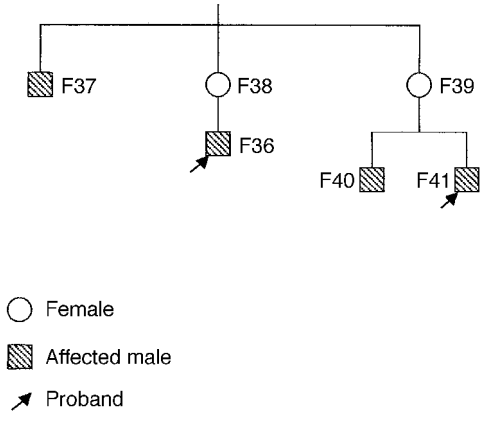

FIGURE 1. *The pedigree chart of family A.*

Southern hybridization

For each sample, 10 μg of genomic DNA was digested with *Eco*RI (Genei, Bangalore, India) for the detection of amplification of CGG repeats. To study the methylation pattern we used double digestion of 10 μg of DNA with *Eco*RI (Genei) and *Eag*I (Arlington Heights IL, USA) as suggested by Rousseau *et al.*[11] In each case the products were run on a 0·8% agarose gel. The gels were processed, set up for Southern transfer and the positively charged membranes were hybridized using the methods described by Rousseau *et al.*[11]

Cytogenetic analysis

About 5 mL of venous blood was drawn from patients and 0·5 mL of leucocytes was inoculated in folate-deficient medium 199 to induce the fragile sites, following the method of Brookwell *et al.*[12] Metaphase chromosomes were prepared and processed for solid staining using the protocol described by Rooney and Czepulkowski.[13] About 100 metaphases were scored per patient and those with fragile sites were photographed. The chromosome with a fragile site was identified as the X chromosome using a G-banding technique adapted from Rooney and Czepulkowski.[13]

RESULTS

Polymorphism of CGG repeats

Primers C and 571R used in the PCR assay detect 265 base pairs of unique sequences in

addition to the CGG repeats.[9] Using the Expand™ Long Template enzyme mix, the repeats in the normal range could be easily detected. Premutations were detected in a few cases together with the intermediate alleles (40–55 CGG repeats). Full mutations, however, could not be amplified properly and the products showed smears. In our study, 21 distinct alleles were identified. The modal repeat number was 28 (41·9%) followed by 29 (16·6%). Samples from men showed amplification of one allele; of the 61 women's samples analysed, 40 were found to be homozygous for the CGG repeats. In the remaining 21 women (~34%), two distinct alleles were identified with different CGG repeats and thus they were heterozygous at this locus. Figure 2 shows the CGG repeats in the control DNA samples.

Apart from the detection of normal alleles, the intermediate allele $(CGG)_{43}$ was detected in two men, F41B and N40. Since the amount of amplified DNA decreases with increase in allele size, only premutation alleles in the lower range could be detected. Premutation was seen in three women, N47 (58 and 71 CGG repeats) (*see* Fig. 3, lane 5), F23 (36 and 67 CGG repeats) (*see* Fig. 5a) and F39 (28 and 167 CGG repeats) (*see* Fig. 4a, lane 5). The premutation alleles of N47 could be detected by PCR.

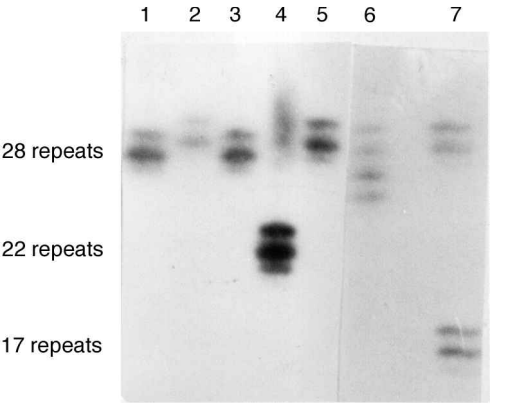

FIGURE 2. *CGG repeat numbers in seven normal individuals. ^{32}P-labelled polymerase chain reaction products were analysed by electrophoresis on a denaturing 6% polyacrylamide gel and visualized by autoradiography. Lanes 1–5 represent samples from five men. Lanes 6 and 7 are samples from women heterozygous for the CGG repeats. The darkest band was used to calculate the size of the fragment.*

Southern hybridization

The probe StB12.3,[7] kindly provided by Prof. J L Mandel (INSERM, France), was used to hybridize *Eco*RI-digested and *Eco*RI + *Eag*I-double-digested genomic DNA.

Figure 4a is representative of the different hybridization patterns obtained for *Eco*RI-digested DNA of normal, carrier and affected persons. In the normal man, N34, StB12.3 detected a band of 5·2 kilobase (kb) (lane 1). In the woman with premutation, F39, as well as the normal 5·2-kb band, an additional band appeared at about 5·7 kb ($\Delta = 500$; where Δ denotes the increase in CGG repeats in base pairs; here it represents 167 CGGs) (lane 5). F38 was a woman with full mutation. Together with her normal allele of 28 CGG repeats (~5·2 kb), a smear was observed in the range of 5·8–7 kb ($\Delta = 600$–1800; 200–600 CGG repeats), representing somatic heterogeneity of her fully expanded alleles (lane 4). Full mutations were observed in the range 6–9·4 kb ($\Delta = 800$–4200; 267–1400 CGG repeats) in the men F36, F37 and F41 (lanes 2, 3 and 6, respectively). Multiple bands appeared in F36 and F37 because full mutations are unstable in somatic cells.

Figure 4b represents the hybridization pattern of F45, where a 6·5-kb band has replaced the 5·2-kb band found in normal men. F45 had one full mutation allele of 433 CGG repeats and the band appeared in the form of a smear due to somatic heterogeneity (lane 2).

Figures 5a and 5b represent the methylation patterns of patients obtained by hybridizing *Eco*RI + *Eag*I-digested genomic DNA with StB12.3. We studied the methylation status in the full mutation individual F40 and the premutation woman F23. Figure 5a represents the methylation pattern of F23, where the four possible combinations of normal active (2·8 kb), premutated active (3 kb, $\Delta = 200$; ~67 CGG repeats), normal inactive (5·2 kb) and premutated inactive (5·4 kb, $\Delta = 200$; ~67 CGG repeats) X chromosome fragments were seen. Figure 5b shows the methylation pattern of the full mutation man, F40, where a 9·4-kb band ($\Delta = 4200$; 1400 CGG repeats) was produced on *Eco*RI + *Eag*I digestion of his DNA sample (lane 2). Lane 1 shows the methylation pattern observed for the normal man, N34, where a 2·8-kb fragment was produced due to his unmethylated X chromosome. Lane 3 shows the normal 5·2-kb band of N34 produced on digesting his DNA sample with *Eco*RI only.

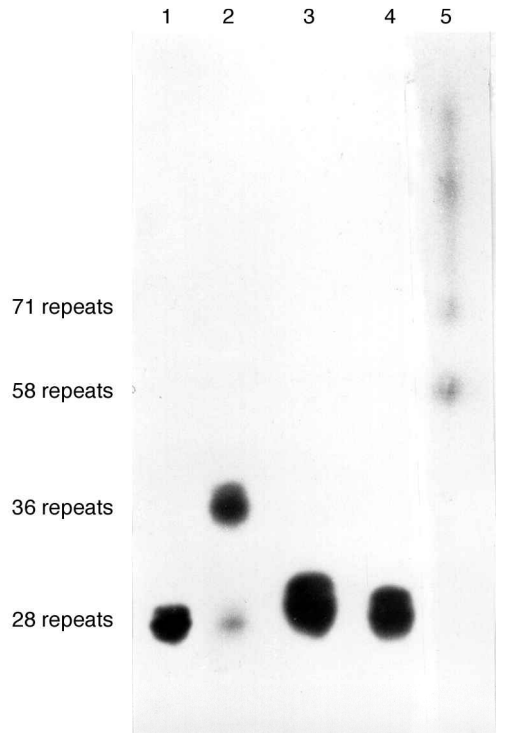

FIGURE 3. *Polymerase chain reaction amplified fragments of CGG repeats analysed on a denaturing 6% polyacrylamide gel and visualized by autoradiography. Lanes 1–4 show normal CGG repeats in four samples from a man and three women, respectively. Lane 5 shows the premutation alleles of the woman N47.*

Cytogenetic studies

Metaphase preparations from leucocytes of patients were analysed to detect fragile sites. On scoring about 100 metaphases, 5–6% cells showed the fragile site at Xq27.3. The metaphase plate of patient F45 is shown in Fig. 6.

DISCUSSION

Variations of CGG repeats at the *FMR1* locus in normal subjects

The CGG repeat sequence involved in the manifestation of the fragile X syndrome shows variation in normal individuals as well as in fragile X patients. The four-allele model proposed by Morton and Macpherson[14] describes four distinct ranges of CGG repeats: normal, <40; intermediate, 40–55; premutated, 56–200; mutated, >200. Among the 21 different alleles observed in our study, $(CGG)_{28}$ appeared to be the most frequent allele in our population, $(CGG)_8$ the smallest and $(CGG)_{43}$ the largest allele observed in the normal individuals.

In a study by Fu *et al.*[9] involving the four races Caucasians, Hispanics, Blacks and Asians, $(CGG)_{29}$ appeared to be the most prevalent allele. A study by Arinami *et al.*,[15] on non-retarded Japanese subjects, showed $(CGG)_{28}$ to be the most frequent allele in their population, and reports[16–18] on the Chinese population showed the most frequent allele to be $(CGG)_{29}$ among their normal subjects. A similar distribution pattern was observed by Richards *et al.*[19] in a study on the different ethnic populations of the world. A report from the southern part of India by Baskaran *et al.*[20] identified $(CGG)_{28}$ and $(CGG)_{31}$ as the most frequent alleles in their population. Figure 7a shows the distribution pattern obtained for our population. Figure 7b compares the allele frequencies of our population with those of Caucasians, Chinese, Japanese and South Indians. There is apparent similarity in the modal repeat number of $(CGG)_{28}$ between our population and those studied by Arinami *et al.*[15] and Baskaran *et al.*[20] If we compare our allelic distributions with the populations mentioned above (*see* Fig. 7b), similarities as well as differences are seen which can be presumed to be due to racial or ethnic influence.

Premutations, full mutations, mosaicism and methylation

Premutations are liable to further expansions when maternally transmitted and the transition is prezygotic.[21] Fu *et al.*[9] related the size of the premutation allele to the risk of expansion to full mutation in maternal transmissions and showed that the risk varies from 0% in the 50–59 repeat range to 100% above 98 repeats. In our study, family A is a good example of the expansion of maternally transmitted alleles. The pedigree chart of the family is shown in Fig. 1. The brother F37 and his two sisters, F38 and F39, inherited expanded CGG repeats in both the premutation and the full mutation ranges. In the woman F39, in addition to her normal allele of 28 CGG repeats, a premutated allele of 167 CGG repeats was observed in the Southern blot (Fig. 4a, lane 5). The premutated allele expanded to full mutation alleles in her sons, F40 (Fig. 5b, lane 2) and F41 (Fig. 4a, lane 6), where alleles of 1400 and 600 CGG repeats were detected, respectively. The other sister, F38, was a carrier of a full mutation together with a normal allele. Because of extensive somatic heterogeneity in her full mutation allele, the band appeared as a

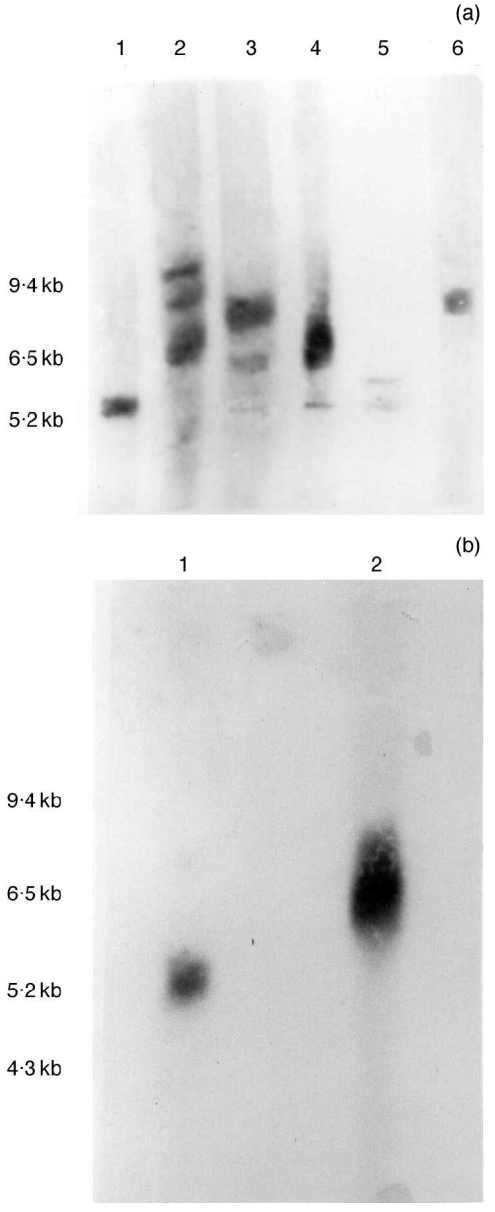

FIGURE 4. (a) *Southern blot analysis of samples from family A.* Eco*RI-digested genomic DNA was hybridized to probe StB12.3. Lane 1 shows the control band for a normal man, N34. Lanes 2–4 and 6 show bands for the full mutation alleles in F36 (male), F37 (male), F38 (female) and F41 (male), respectively. Lane 5 shows the 5·7-kb band for the premutation allele in woman F39. Locations of DNA size markers from Lambda* Hind*III standard are shown on the left.* (b) *Southern hybridization pattern of the male patient F45. Lane 1 shows the control band in a normal man, N24, and lane 2 shows the band for the expanded allele of patient F45.*

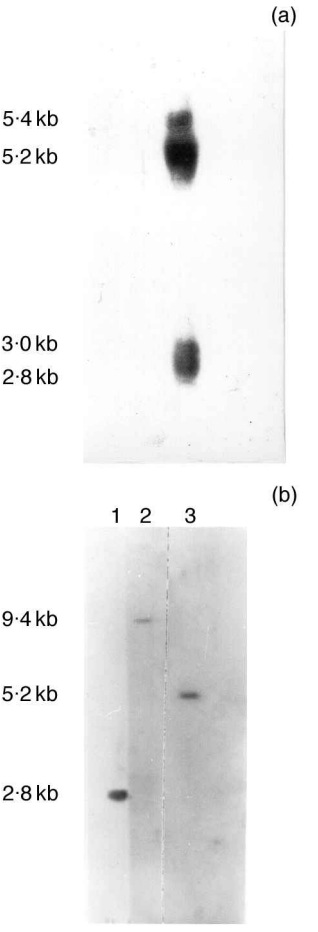

FIGURE 5. (a) *Southern hybridization blot showing the methylation pattern of the premutation woman F23. Shown are the bands for the four possible combinations of normal active (2·8 kb), premutated active (3 kb), normal inactive (5·2 kb) and premutated inactive (5·4 kb) X chromosomes. The size locations are indicated on the left.* (b) *Southern hybridization patterns of* Eco*RI*+Eag*I- and* Eco*RI-digested genomic DNA. Lane 1 shows the band for the unmethylated normal allele in the control man, N34, and lane 2 shows the band for the methylated full mutation allele in subject F40, produced by* Eco*RI*+Eag*I digestion of their DNA samples. Lane 3 shows the control band in the normal man, N34, produced on* Eco*RI digestion of his genomic DNA sample.*

smear in the range 5·8–7 kb, representing CGG repeats in the range of 200–600 repeats (Fig. 4a, lane 4). Her severely retarded son, F36, showed discrete bands of expanded alleles of 600, 933 and 1400 CGG repeats (Fig. 4a, lane 2).

FIGURE 6. *The metaphase plate of sample from subject F45, showing solid stained chromosomes. The arrow indicates the fragile site on the X chromosome.*

Mosaicism has been described as the co-existence of 'full mutation' and 'premutation' alleles,[9] and an estimated 12–41% of affected men are mosaics.[22] The presence of a normal-sized allele together with a full mutation was described in a mosaic patient by Orrico *et al.*[23] and recently by Schmucker *et al.*[24] We have made similar observations in the male subject F37, where mosaicism appeared not only with respect to full mutation (6·9–7·4 kb; 600–933 CGG repeats) and premutation (5·8-kb band; 200 CGG repeats) but also with the presence of a faint band of 5·2 kb (*see* Fig. 4a, lane 3). A significant level of mosaicism was also seen in the case of F38 and her son F36 as described earlier.

As described above,[2,11] methylation yields very distinctive patterns in men and women

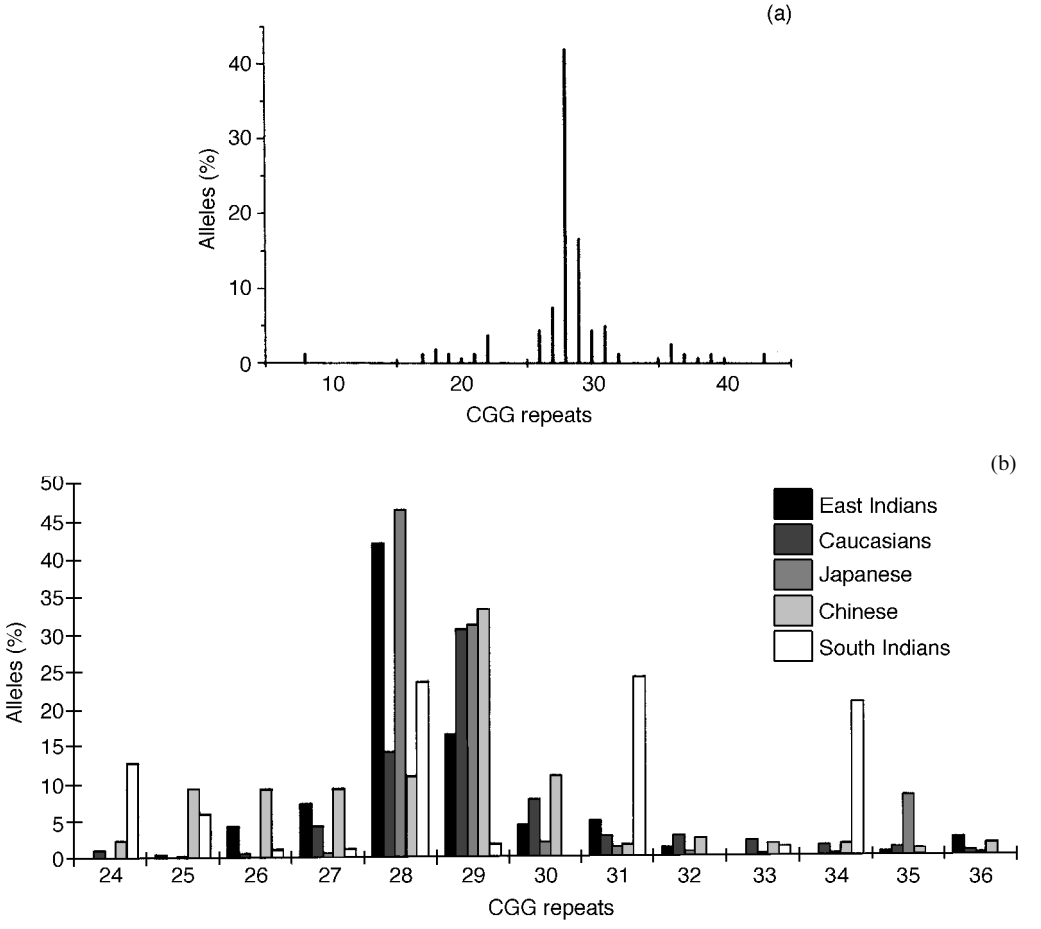

FIGURE 7. (a) *Distribution of the allele frequencies for the polymorphic CGG repeat in the* FMR1 *gene in our studied population.* (b) *Histogram showing the distribution and comparison of a selected range of CGG repeats in Caucasians, non-retarded Japanese, Chinese from South China, South Indians and our studied population.*

when they are carriers of premutation and full mutation. By digesting DNA samples simultaneously with *Eco*RI and *Eag*I, amplification as well as methylation can be studied. We studied a woman, F23 (*see* Fig. 5a), who had developmental delay and severe psychiatric abnormalities. Analysis of her DNA sample by PCR revealed a normal allele of 36 CGG repeats and a premutation allele of 67 CGG repeats. This was confirmed by hybridizing her DNA sample with StB12.3 after digestion with *Eco*RI + *Eag*I. Premutation was observed on her X chromosomes, represented by the typical four-band pattern due to random X inactivation in her cells as described by Rousseau *et al.*[11] The findings in individuals with developmental delay who are found to carry only a premutation are difficult to interpret. The presence of a premutation may be an indicator of full mutations in other tissues.[22,25] We also observed the methylation pattern of a normal man, N34 (*see* Fig. 5b, lane 1), where the unmethylated normal X chromosome produced a 2·8-kb band as expected. The methylation pattern of a man with full mutation, F40 (*see* Fig. 5b, lane 2), showed the presence of a 9·4-kb band, suggesting an expansion of 1400 CGG repeats and subsequent inactivation of the X chromosome.

In the present study, among 98 cases of unrelated mental retardation patients, six men and one woman were found to be affected with full mutations, representing a frequency of 7%. This frequency is consistent with the frequencies observed by Maino *et al.*[26] and Brown,[27] which ranged from 2% to 7%. Among the 61 women studied, four premutation alleles were detected. Of these, two were mothers of suspected fragile X patients, one had psychiatric abnormalities and one was an apparently normal woman. Rousseau *et al.*[28] reported the frequency of premutation to be 1:500 X chromosomes in a large group of samples from women. However, our sample size is too small to draw any such conclusion about the frequency of occurrence of premutation in our population. In all the fragile X patients we have observed, the expansion of CGG repeat and the hybridization patterns do not suggest the presence of any deletion in the *FMR1* gene.[29–31] As the clinical status of the patients could be correlated with the classical expansion of the CGG repeat these samples were not tested for the presence of point or frameshift mutations that disrupt the *FMR1* gene structure and render it non-functional.[32] This suggests that the hyperexpansion of the CGG repeat is the overwhelmingly frequent cause of the syndrome in our population, although the possibility of other types of mutations in the *FMR1* gene cannot be ruled out.

The methylation status of the CpG island near the CGG repeat sequence influences the expression of the *FMR1* gene and can be studied using the methylation-sensitive restriction enzyme *Eag*I.[7,11] We have observed the methylation pattern on a normal man, a man with full mutation and a woman with premutation. However, we could not study the methylation status of the mosaic individuals due to paucity of the DNA samples; this study should be carried out, to ascertain the situation in these individuals. Also, we restricted our study to blood leucocytes. Since the syndrome has tissue-specific variations, DNA samples from other tissues such as fibroblasts should also be studied in order to obtain a clearer picture of the molecular nature of the syndrome.

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