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Fragile X Allelemorphism among the Mentally Retarded and Affected Families

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Abstract: The fragile X mental retardation allele was investigated in Ibadan, south west Nigeria. Blood specimens from a population of 659 Mentally Retarded individuals (MRs) were screened for the fragile X mutation using cytogenetic and molecular methods. It was observed that 235 (35.7%) individuals had chromosomal aetiologies to their mental impairment. The Down syndrome was highest occurring in 146 (21.2%) individuals, followed by the fragile X karyotype with 45 (6.83%) individuals expressing the 46, Xq 27.3 fragility. Of the fragile X individuals, there were 13 (2.0%) fragile X females, 2 of which were 45, XO/46, XX and 45, XO/46, XX/47, XXX mosaics, respectively. Molecular methods confirmed the cytogenetic findings, where affected individuals expressed the of trinucleotide repeat amplification in the order of >200 CGG repeats in the fragile X allele region. Triplet repeat bands ranged between 200 and 2000 CGGs. Eight pedigrees comprising 70 normal relatives of 8 fragile X mentally retarded propositi were permissible to investigations for the determination of interfamilial transmission of the fragile X alleles. Blood samples were equally obtained from them and analysed, using cytogenetic and molecular methods likewise. Two normal sisters of a male propositus exhibited the 45, XO/47, XXX and 46, Xr(X)/45, XO mosaicisms, respectively. Molecular analysis revealed 26 (33.3%) female permutation carriers and 11 (14.1%) normal transmitting males. Eighteen (23.1%) males had normal alleles thus non-transmitting males and 15(19.2%) females were normal. The proportions observed in this survey, has implications for the general population and should prove significantly useful for clinicians and genetic counsellors.

Key words: Fragile X syndrome, mental retardation, karyotype, femal carriers, molecular analysis

INTRODUCTION

The fragile X syndrome (FSX) was the first example of a class of human genetic mutations to be identified which involve amplification of triplet repeats and show anticipation that is, increasing severity or frequency of the disorder within sibships over succeeding generations (De Vries *et al*., 1998). Current estimates, though somewhat lower than previously reported indicate approximately 1 in 4000 males, 1 in 6000 females in the general population are retarded as a result of the syndrome (Van Esch, 2006) and 1 in 600 females is a carrier (Donnenfeld, 1998). FXS is still the highest cause of inherited Mental Retardation (MR) and accounts for perhaps 2 and 4% of mental retardation overall. It is second only to Down syndrome as a specific cause of MR (Brown, 1995). The designation fragile X is based on the presence of a gap or break at the end of the on arm of the X chromosome when affected cells are cultured in special media (Sutherland *et al*., 1985). Approximately, 95% of adult fragile X males have an IQ below 70 with overall mean of 35 (Shiue *et al*., 2004; Sutherland *et al*., 1985) Fragile X syndrome also appears to show significant association with infantile autism (Belmonte *et al*., 2004; Blanc *et al*., 1993). Autistic individuals are usually males and a 4:1 male to female ratio is commonly observed. About one in 5 males who inherit the fragile X chromosome are normal carrier males (Sherman *et al*., 1985). These males are non-penetrant for the mutation and do not express the fragile site. They transmit the mutation to their daughters who are also non-expressing for the mutation but who can then have affected sons. Majority of female permutation carriers are mentally normal. Approximately one in 3 females have some evidence of mental impairment and about one in 10 is considered mentally retarded. A characteristic profile of cognitive defects in affected carriers may be present (Brown, 1995). The syndrome and its particular pattern of heredity are caused by a dynamic mutation, involving an unstable expansion of a trinucleotide (CGG) repeat at the 5' UTR of the FMR1 gene, located at Xq 27.3. Expansion of this repeat region greater than 200 repeats leads to methylation-coupled silencing of the gene and absence of the Fragile X Mental Retardation Protein (FMRP), causing

the classical FXS (Van Esch *et al*., 2005). Individuals with expanded repeat lengths varying from 50-200 repeats do not exhibit the classical FXS phenotype, but are considered as fragile X premutation carriers (Van Esch *et al*., 2005).

The FMR1 gene is expressed early in development. With maturation, it is expressed strongly in the brain, gonad, oesophagus, thymus, spleen and lymphocytes, weakly in the liver, kidney, colon, thyroid and lung, but not at all in muscle, heart and aorta (Hinds *et al*., 1993).

Although, studies on the FXS abound in literature from many populations, the author is oblivious of any prior publication on the prevalence of the FXS from a Negroid African cohort population. This might be due in part to the fact that compared to individuals diagnosed with most other genetic or chromosomal syndromes, affected fragile X males usually are fairly normal in physical appearance and therefore escape detection earlier in life. This study reports, the prevalence of the FXS among Mentally Retarded individuals (MRs) and examines the intra-familial dynamics of the FMR1 allele transmission.

MATERIALS AND METHODS

Subjects: Mentally Retarded individuals (MRs) were sought from schools/homes for the mentally handicapped in the city of Ibadan and environs. The criteria for recruitment into this study were mental retardation and evidence of registration in these special schools/homes for the mentally retarded. Peripheral blood specimen was obtained via vene-puncture aseptically from the MRs in a continuous exercise and as they become available between 1996 and 2001. All subjects ranged from mildly to severely retarded. Equal volume of blood was withdrawn from mentally normal individuals of similar age distribution and used as controls.

Familial investigation was routinely sought from all FXS individuals and where permissible blood specimen was withdrawn from volunteer family members. The criterion for inclusion in this part of the study was familial relationship.

Constitutive karyology: Phytohaemaglutinin (PHA) stimulated whole blood microculture technique (modification of the technique of (Jacky *et al*., 1991) was used to cultivate the lymphocytes of each donor. About 0.2 mL whole blood inoculum was injected into 5 mL of medium 199 for fragile X (sigma) supplemented with pooled human AB + ve serum, gentamicine, L-glutamine and PHA. Cultures were incubated at 37° for 72 h and harvested. Vincristine sulphate (a spindle inhibitor) was introduced at 70 h (0.002 μg mL^{-1} of culture) and at 72 h cultures were spun down at 5000 rpm for 3 min. The pellets were hypotonised in 0.075M KCL solution for 8 min spun down again and re-suspended in fresh cold fixative (3:1 methanol: glacial acetic acid). Slides were prepared and analysed microscopically. A minimum of 50 non-banded Giemsa stained cells and 10 Giemsa-trypsin banded cells were scored for the fragile X. Numerical aberrations encountered were recorded and banding was used primarily to distinguish the X-chromosome from other group C chromosomes.

DNA isolation: Blood spotted on blotting papers and dried were lysed in Sodium Dodecyl Sulphate (SDS)-perchlorate, digested overnight with proteinase K and isolated according to the procedure of (Strauss, 1988). The DNS from all samples was extracted with chloroform and precipitated with cold ethanol.

PCR amplification: Amplifications were carried out in a thermalcycler in 40 μL reactions, containing buffer (10 mmol L^{-1} tromethamine hydrochloride, pH 8.3, 50 mmol L^{-1} potassium chloride), 1.25 U Taq polymerase, 10% dimethyl sulfoxide (DMSO), 25 pmol of each primer and approximately 200 ng of template DNA. The dNTPs were 200 μmol L^{-1} eash of dATP, ACTP, DTTP and 7-deaza-2-deoxyguanosine triphosphate (7-deaza-dGTP). Magnesiun chloride concentration used was 1.25 mmol L^{-1}.

The PCR primers used were (5'-GAC GGA GGC GCC GCT GCC AGG- 3') (invitrogen) for the forward amplification and (5'-TCC TCC ATC TTC TCT TCA GCC CT -3') (invitrogen) for the reverse amplification. The primers annealed at 61°C. Denaturations were performed at 94°C to optimise product yield of disparate size alleles (Walsh *et al*., 1992).

Electrophoresis and gel transfer: About 5uL of PCR product were combined with 5uL of loading buffer (80% formamide, 0.1% bromophenolblue, 0.1 Xylene cyanol) and separated by electrophoresis through 6% polyacrilamide, 8.3 mol L^{-1} urea denaturing gel with TBE running buffer at 500 V for 60 min, then transferred to positively charged nylon filters by electrblotting (Trnovski, 1992) for 30 min using semi dry electroblotter at 3 mA cm^{2-1} constant current.

Chemilumiscence probe analysis: For the detection of PCR products, a hybridising oligonucleotide probe producing chemiluminiscence was used

(Pollard-Knight *et al.*, 1990). Probe, chemiluminiscent spray and hybridising agents were supplied as a kit (invitrogen). A (CGG)-5' probe labelled with alkaline phosphatase was hybridised to the nylon membranes for 20 min at 55°C, sprayed with chemiluminiscent spray, sealed in plastic folders and exposed to roentgenogram film at 37248°C for 60 min and overnight.

RESULTS

The MRs studied was aged between 12 and 39 years old and they ranged between mildly to severely retarded. They all attended special schools/homes for the mentally handicapped. A total of 659 samples collected over a 5 year period (1996-2001) were screened cytogenetically for the fragile X chromosome. The summary of the cytogenetic result presented in Table 1 showed that 32.6% had chromosomal aneupliodies as the aetiologies of their conditions. Down syndrome was highest in occurrence among the MRs of 22.2%, followed by the FXS with a frequency of 6.8% individuals expressing the Xq27.3 X-chromosome fragility. Two (15.4%) FXS females exhibited cytogenetic mosaicisms of 45XO/46XX and 45XO/46XX/47XXX karyotypes respectively.

In pedigree studies, 8 probands and 70 members of their respective families were permissible to investigations (Fig. 1-8). These 70 volunteers were mentally normal relatives of 4 mentally retarded FXS males and 4 mentally retarded FXS females. The cytogenetic data of the 8 pedigrees as well as their fragile X allele status is presented in Table 2. Two normal sisters of a male proband in family H (Fig. 8) were observed to exhibit cytogenetic mosaicism one of whom was a numerico-structural mosaic of the 46Xr(X)/45/XO karyotype.

Table 1: Summary of fragile X allele prevalence and cytogenetic status of the mentally retarded individuals

Status	No. of males (%)	No of females (%)	Total
(a) CGG repeat			
Normal	349 (53.0)	222 (33.7)	571
Premutation carriers	7 (1.06)	16 (2.43)	23
Affected MR	52 (7.90)	13 (1.97)	65
Total	408	251	659
(b) Karyotypic			
46, XY	206 (31.3)	-	206
46, XX	-	238 (36.1)	238
46, XY fragile Xq27.3	32 (4.90)	-	32
46, XX fragile Xq27.3	-	11 (1.67)	11
47, XX+G21	-	59 (8.95)	59
47, XY+G21	87 (13.2)	-	87
47, XXY	7 (1.06)	-	7
45, XO	-	17 (2.58)	17
45, XO/46,XX fragile Xq27.3	-	1 (0.15)	1
45, XO/46, XX/47, XXX fragile Xq27.3	-	1 (0.15)	1
Total	332	327	659

Table 2: Summary of fragile X allele and cytogenetic status in the pedigrees

Status	No of males (%)	No. of females (%)	Total
(a) CGG repeat			
Normal	23 (29.5)	16 (20.5)	39
Premutation carriers	5 (6.41)	26 (33.3)	31
Affected MR	4 (5.10)	4 (5.10)	8
Total	32	46	78
(b) Karyotypic			
46, XY	28 (35.9)	-	28
46, XX	-	40 (51.3)	40
45, XO/46,XX	-	1 (1.30)	1
45, XO/46, XX/47, XXX	-	1 (1.30)	1
46, XX/47, XXX	-	1 (1.30)	1
46, Xr(X)/45, XO	-	1 (1.30)	1
46, XX fragile Xq27.3	-	2 (2.56	2
46, XX fragile Xq27.3	4 (5.1)	-	4
Total	32	46	78

Table 3: Composition of the fragile X allele status in the pedigrees

Pedigree	No. of Normal males	No. of carrier females	No. of affected males	No. of normal females	No. of carrier females	No. of affected females	Total indivi-duals
Fig. 1	4	1	-	3	2	1	11
Fig. 2	3	2	-	1	4	1	11
Fig. 3	-	1	1	2	3	-	7
Fig. 4	2	1	1	-	5	-	9
Fig. 5	2	5	1	2	3	-	13
Fig. 6	1	-	1	4	1	-	7
Fig. 7	4	-	-	3	2	1	10
Fig. 8	1	1	-	-	7	1	10
Total	17	11	4	15	27	4	78

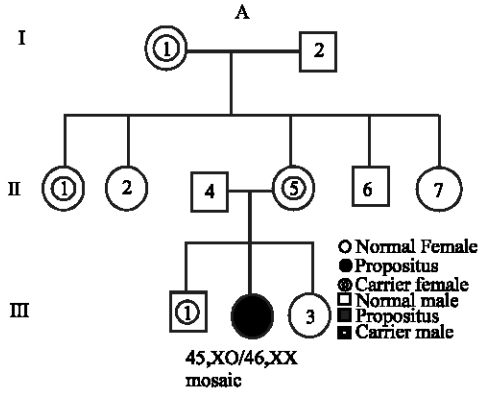

Fig. 1: Family A with female mosaic propositus who had a normal father, a carrier mother and a normal grandfather

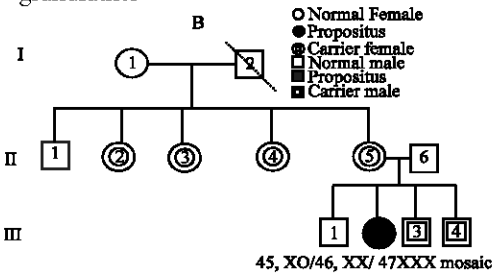

Fig. 2: Family B with female mosaic propositus who had a normal father, a carrier mother and two normal transmitting brothers

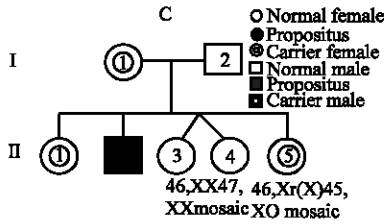

Fig. 3: Pedigreeof family C with a male prispositus who had two mentally narmal cytogenetically mosaic sisters, a normal transmitting father and a carrier mother

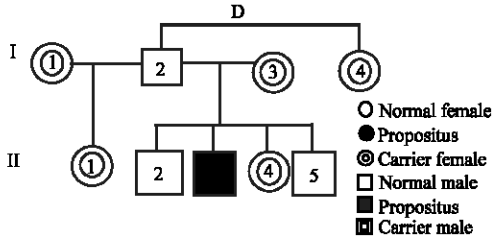

Fig. 4: Pedigree of family D with a male prispositus who had a normal transmitting father and a carrier mother

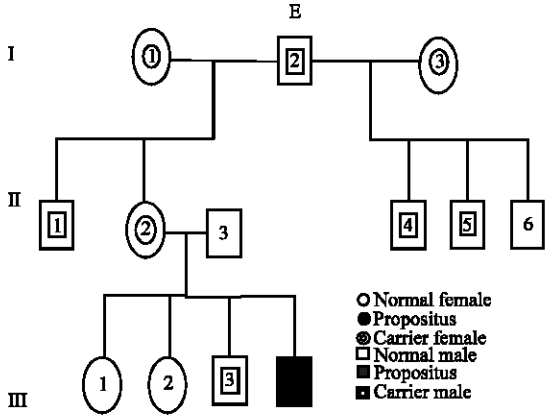

Fig. 5: Family E with male propositus who had a normal non-transmitting father and a carrier mother and a normal transmitting grandfather

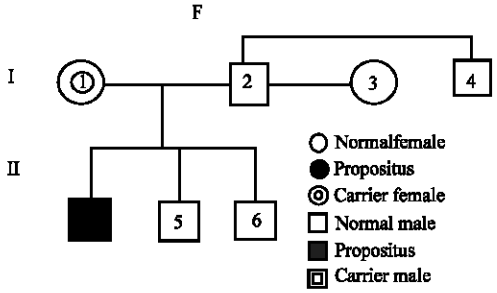

Fig. 6: Family F with male propositus who had a normal carrier mother and a non-transmitting father

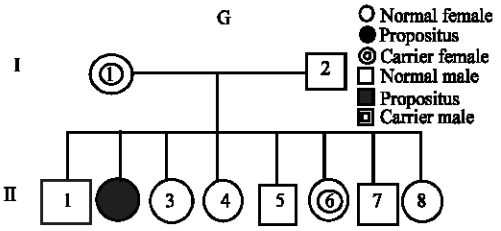

Fig. 7: Family G with male propositus who had anormal carrier mother and a non- transmitting father

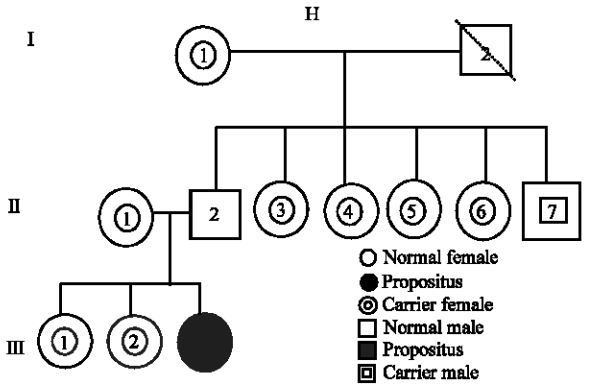

Fig. 8: Family H with male propositus who had anormal carrier mother and a non- transmitting father. There is a preponderance of carriers

Although, other members of the pedigrees appeared to be cytogenetically normal, the molecular methods revealed some interesting features about the transmission pattern of the FMR1 allele. The premutation (PM) carrier status of individuals in the respective pedigrees is shown in Table 3. The phenomenon of anticipation and instability of the CGG repeat expansion with succeeding generation was observed in families D and E (Fig. 4 and 5, respectively). On the pedigree charts (Fig. 1-8), daughters of unaffected transmitting males inherited the PM carrier status of the FXS condition, while males are likely to be affected only when there is an amplification of the repeat sequence trans-generational from their mothers. Daughters of carrier mothers also inherited the PM carrier status but not the full mutation with an exception in family H where there is a normal non-transmitting father and a normal PM carrier mother.

DISCUSSION

The different karyologies observed among this group of MRs (Table Ib) showed the FXS to be second only in frequency to trisomy G-21 (Down syndrome) as the most common cause of mental retardation with chromosomal

aetiology. Screening populations for the fragile X chromosome has yielded heterogeneous results, depending on the selected criteria of subjects. Some studies in male populations selected individuals with macroorchidism for cytogenetic analysis (Brown *et al*., 1981; Howard-Peebles and Finley, 1983; Venter *et al*., 1984). Others selected male populations with familial mental retardation (Fryns *et al*., 1984; Venter *et al*., 1981).

These investigations showed the highest frequencies of males with FXS (Primrose *et al*., 1986). In most such studies the selection criterion was mental retardation not associated with chromosomal aberrations (Arinami *et al*., 1987; Blomquist *et al*., 1983; Sutherland *et al*., 1985; Thake *et al*., 1987). Very few studies however included all individuals in a study group regardless of the cause of mental retardation (Jacobs *et al*., 1986; Kahkonen *et al*., 1986). In the case of males macroorchidism is not always present. The choice of mentally defective population for this study removes this bias and much more random and reliable data could be obtained. The prevalence of FXS in males in institutions for the mentally retarded was 1.6-6.0%, rising to 7-9% if known chromosomal or dysmorphic cases are excluded (Pembrey and Baraitser, 1986) with improved methodologies. In this particular study, the preponderance of males with the FXS among the MRs is 4.9% (Table 1b). The most significant impact of improved diagnostic tool was in the elimination of equivocal fragile X detection results. It is conjectured that since the criterion for screening is solely mental retardation and devoid of any known dysmorphic, chromosomal or familial bias, the proportions of unaffected carrier males and females observed in the pedigrees might have implications for the general population (Table 2).

The observed mosaic karyotypes and gel bands in the pedigree studies is indicative that this phenomenon is not uncommon with the inheritance of the FMR1 alleles. Since, the mutations of the CGG repeats are dynamic and may vary from generation to generation as well as within a single person during early embryogenesis (Devys *et al*., 1992; Wohrle *et al*., 1993), these premutation (PM) alleles may become unstable, only through maternal transmission, with further expansion in the next generations. For a long-time, male and female PM carriers were considered as asymptomatic. This view was, however, gradually challenged with the description and reports of different PM-associated clinical phenotypes over the last decade (Van Esch, 2006).

According to the standard model, the FMR1 gene becomes silenced as a consequence of the expansion and the methylation of the CGG trinucleotide repeat element located in the 5'-UTR of the gene (Pieretti *et al*., 1991). The resulting absence (or deficit) of the Fragile X Mental Retardation Protein (FMRP) causes FXS. Based on this model, carriers of the PM alleles were assumed clinically normal (asymptomatic), since methylation-coupled silencing does not occur in the PM range. However, contrary to expectation, a subgroup of male and female carriers does show some clinical involvement which includes physical features, learning disabilities, anxiety, developmental delay and emotional problems. In addition, approximately 20% of female carriers of PM alleles have premature ovarian failure, which is not observed in females with hypermethylated full mutation (Allingham-Hawkins *et al*., 1999). This may explain the condition of the proband in family H (Fig. 8) who did not express the fragile X cytogenetically, but a mentally retarded PM carrier; who also suffers from ambiguous external genitalia (unpublished observation). Non-expressing males do not express the fragile site, they transmit the mutation to their daughters who are also none expressing for the mutation but can have affected sons. This pattern follows through in the pedigrees where the inheritance of the fragile-X chromosome descended from maternal grandfathers. Apparently males do not inherit any X chromosome paternally and as such will not be expected to be carriers if their mothers carry normal alleles.

CONCLUSION

The occurrence of mosaicism in female subjects in this survey is not unexpected. Mosaicism is correlated with high functioning and since FXS is a sex linked condition, mosaicism when it occurs, show a strong pattern.

The pattern of the fragile X inheritance and the behaviour of the alleles will suggest pertinence to genetic counselling particularly in families with high female PM frequencies. Further work with larger sample size and pedigrees with large kindred should prove useful in extending the reliability of our indices for risk evaluation in the general populations.

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