European Journal of Human Genetics (1998) 6, 518–522
© 1998 Stockton Press All rights reserved 1018–4813/98 $12.00
http://www.stockton-press.co.uk/ejhg

SHORT REPORT

Analysis of *FMR1* and flanking microsatellite markers in normal and fragile X chromosomes in Portugal: evidence for a "protector" haplotype

Ana Peixoto[1], MR dos Santos[2], Raquel Seruca[1], António Amorim[1,3] and Sérgio Castedo[1,4]

[1]*Instituto de Patologia e Imunologia Molecular da Universidade do Porto*
[2]*Instituto de Genética Médica Dr Jacinto Magalhães, Porto*
[3]*Faculdade de Ciências, Porto*
[4]*Serviço de Genética Médica, Faculdade de Medicina, Porto, Portugal*

In order to look for linkage disequilibrium between the fragile X locus and its flanking markers, we analysed the FRAXAC1 and DXS548 microsatellites in normal and fragile X individuals of Portuguese origin. We observed differences in allele and haplotype frequencies between these two samples. Four haplotypes (A-2, C-2, C-5 and D-6) accounted for 76% of all fragile X chromosomes, whereas a single haplotype (C-7) accounted for 70% of the normal population and less than 3% of the fragile X chromosomes. Among the four observed high-risk haplotypes, A-2 and D-6 had been previously reported in other studies, but C-2 and C-5 seem characteristic of Portuguese patients, as suggested by the high frequency (38%) in fragile X chromosomes and virtual absence in controls. In accordance with previous studies, a greater heterozygosity of the fragile X sample was noted when compared to that of controls. The high frequency of C-7 haplotype in the normal population and its virtual absence in the fragile X sample may reflect the existence of linkage disequilibrium between the two loci and/or selective advantage (protector effect) of this haplotype.

Keywords: fragile X syndrome; microsatellites; FRAXAC1; DXS548; founder effect; Portugal

Introduction

The fragile X syndrome is the most frequent cause of familial mental retardation with an estimated prevalence of about 1:4000 males and 1:8000 females.[1] It is an X-linked dominant disorder with reduced penetrance. The clinical aspects of the fragile X syndrome in adult male patients are moderate to profound mental retardation, long and narrow face, prominence of the jaw and forehead, large ears and macro-orchidism.[2] This syndrome is associated with a fragile site (FRAXA) on the X chromosome at Xq27.3.

Correspondence: Prof Dr Sérgio Castedo, IPATIMUP, Rua Dr Roberto Frias s/n, 4200 Porto, Portugal. Tel: +351 2 5570700; Fax: +351 2 5570799
Received 10 December 1997; revised 26 January 1998; accepted 5 February 1998

The fragile X syndrome is associated with amplification of a CGG repeat sequence in the 5' untranslated region of the *FMR1* gene.[3–5] The number of repeats is polymorphic in normal individuals, usually in the range of 6–60 triplets (with a mode of 30), frequently interrupted by 1–3 AGG triplets.[6–8] Premutation alleles of nonpenetrant carriers of the fragile X syndrome have between 60 and 200 repeats, which tend to increase in size when maternally transmitted to the offspring. The expansion of fragile X premutation alleles to a full mutation (CGG > 200) occurs exclusively during female transmission. The full mutation is associated with the hypermethylation of the *FMR1* CpG island located 250 bp upstream, which silences gene expression, thus preventing protein production.[9,10] Only the full mutation is associated with clinical and cytogenetic expression of the fragile X syndrome.[11]

The molecular mechanisms responsible for the expansion of these triplet repeats are still not known. As yet, no conversion of normal to premutation alleles has been documented. This might be due to ascertainment bias or to the fact that carriers of premutation have no phenotypic manifestation and can persist for several generations before undergoing transition to full mutation. The absence of documented *de novo* mutations among fragile X families, despite the high prevalence of this disorder, is in line with the possibility of a founder effect, as suggested by the first observation of significant linkage disequilibrium between the fragile X locus and flanking polymorphic markers.[12]

In order to search for linkage disequilibrium in the Portuguese population we compared the allele distribution of markers FRAXAC1 and DXS548 in two samples of normal and fragile X chromosomes.

Materials and Methods

The patient population included 42 unrelated males with documented fragile X syndrome. Samples from 86 unrelated blood donor males were used as controls of the general population. All individuals in this study were of Portuguese origin.

Genomic DNA was extracted from peripheral leukocytes according to standard procedures.[13] All samples referred for fragile X syndrome were screened for the presence of the amplification of the CGG repeat and methylation of the CpG island in the *FMR1* promoter by Southern blot.[14] Two polymorphic CA repeats, one (FRAXAC1) 7 kb and the other (DXS548) 150 kb proximal to the CGG sequence, were used to haplotype normal and fragile X chromosomes. The two markers were amplified using previously described primers.[4,15] PCR reactions were carried out in 20 μl containing 50–100 ng genomic DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dTTP, 200 μM dATP, 200 μM dGTP, 20 μM dCTP, 1 μCi α-(^{32}P)-dCTP, 100 ng primer and 0.5 units Taq polymerase (Pharmacia). After an initial denaturation (4 min at 94°C), 30 cycles (1 min at 94°, 1 min at 59°, 1 min at 72°) were employed for amplification, followed by a 10 min hold at 72°C in a Perkin-Elmer 9600 GeneAmp PCR system. The products were electrophoresed on 6% polyacrylamide sequencing gels. Gels were dried and exposed to X-ray film for 4–5 h.

Statistical analysis was performed using χ^2 distribution tests. The χ^2 was only calculated for cases where expected values were above 5.

The heterozygosity was calculated according to Nei.[16]

Results

Altogether, 86 controls and 42 fragile X chromosomes were analysed and the allele frequencies of the two markers FRAXAC1 and DXS548 in the control and fragile X populations are summarised in Table 1 and Table 2, respectively. Four different alleles were detected at the FRAXAC1 locus and 8 different alleles at the DXS548 locus. χ^2 distribution tests demonstrated significant differences between frequencies of the FRAXAC1 alleles C and D, with C being enriched among the controls (71% *vs* 40% in fragile X chromosomes) and D in the fragile X sample (38% *vs* 17% in normal chromosomes). Despite the higher frequency of

Table 1 FRAXAC1 alleles on control and fragile X chromosomes. Percentage values are indicated in brackets

Alleles	*Controls*	*Fra(X)*	P-*value*[a]
FRAXAC1			
A	9 (10.5)	9 (21.4)	0.050 < P < 0.100
B	1 (1.2)	0 (0.0)	
C	61 (70.9)	17 (40.5)	P < 0.001
D	15 (17.4)	16 (38.1)	0.010 < *P* < 0.025
Total	86	42	

[a]Calculated using χ^2 distribution tests.

Table 2 DXS548 alleles on control and fragile X chromosomes. Percentage values are indicated in brackets

Alleles	*Controls*	*Fra(X)*	P-*value*[a]
DXS548			
1	3 (3.5)	0 (0.0)	
2	6 (7.0)	15 (35.7)	*P* < 0.001
3	1 (1.2)	0 (0.0)	
5	1 (1.2)	8 (19.0)	
5.5	0 (0.0)	1 (2.4)	
6	7 (8.1)	9 (21.4)	0.025 < *P* < 0.050
6.5	1 (1.2)	0 (0.0)	
7	67 (77.9)	9 (21.4)	*P* < 0.001
Total	86	42	

[a]Calculated using χ^2 distribution tests.

FRAXAC1 allele A in the fragile X population as compared to controls (21% *vs* 10%, respectively), these differences did not attain the level of statistical significance.

The distribution of DXS548 alleles revealed significant differences between the two populations: most of the normal chromosomes carried the DXS548 allele 7 (78% *vs* 21% on fragile X chromosomes), whereas the fragile X chromosomes showed preferential association with alleles 2 (36% *vs* 7% in controls), 5 (19% *vs* 1% in controls) and 6 (21% *vs* 8% in controls). DXS548 intermediate alleles 5.5 and 6.5 were each observed only once in controls and fragile X chromosomes.

Overall, 16 different haplotypes were observed in control and fragile X chromosomes (Table 3). χ^2 distribution tests showed significant differences between FRAXAC1-DXS548 haplotypes C-7 and D-6 in the two populations. Most of the normal chromosomes had the C-7 haplotype (70% *vs* 2% in fragile X chromosome). In contrast, four haplotypes alone accounted for the majority of the fragile X chromosomes: A-2 (17% *vs* 5% in controls); C-2 (19% *vs* 0% in controls); C-5 (19% *vs* 0% in controls); D-6 (21% *vs* 6% in controls).

The haplotype distribution in the fragile X population was as could be expected from the allele frequencies. On the contrary, in the normal population, the frequency of the C-7 haplotype was higher than that expected from allele frequencies. The heterozygosity values for FRAXAC1 and DXS548 were 45.8% and 38.1% in controls, and 65.1% and 75.5% in the fragile X sample, respectively.

Table 3 FRAXAC1-DXS548 haplotypes on control and fragile X chromosomes. Percentage values are indicated in brackets

Haplotypes	*Controls*	*Fra(X)*	P-*value*[a]
FRAXAC1/DXS548			
A-1	3 (3.5)	0 (0.0)	
A-2	4 (4.7)	7 (16.7)	
A-3	1 (1.2)	0 (0.0)	
A-5.5	0 (0.0)	1 (2.4)	
A-6	1 (1.2)	0 (0.0)	
A-7	0 (0.0)	1 (2.4)	
B-2	1 (1.2)	0 (0.0)	
C-2	0 (0.0)	8 (19.0)	
C-5	0 (0.0)	8 (19.0)	
C-6	1 (1.2)	0 (0.0)	
C-7	60 (69.8)	1 (2.4)	$P < 0.001$
D-2	1 (1.2)	0 (0.0)	
D-5	1 (1.2)	0 (0.0)	
D-6	5 (5.8)	9 (21.4)	$0.005 < P < 0.010$
D-6.5	1 (1.2)	0 (0.0)	
D-7	7 (8.1)	7 (16.7)	$0.100 < P < 0.250$
Total	86	42	

[a]Calculated using χ^2 distribution tests.

Discussion

A high mutation rate has been suggested to explain the high prevalence of the fragile X syndrome despite the reduced reproductive fitness of affected males and females.[17,18] To date, however, *de novo* mutations have not been detected.[19,20] Moreover, significant linkage disequilibrium between the *FMR1* locus and flanking microsatellite markers has now been reported in several Caucasian populations,[12,21–29] thereby suggesting a founder effect in the fragile X syndrome. This represents a very unusual finding for an X-linked disease with high prevalence and in which affected males rarely reproduce. Morton and Macpherson proposed a multistep model with four different types of alleles for the fragile X locus.[30] According to this model, a mutated allele, S (> 50 CGG repeats) would originate from a normal stable allele, N. S alleles would have a high prevalence in the population and could be maintained for as many as 90 generations, after which they could give rise to an unstable allele, Z (premutation). The full mutation, L, would arise from Z at a remarkably high rate, requiring a female germline transmission. This model was the first to provide an explanation for the possible coexistence of a high frequency of the mutation and a founder effect.

Using the combination of the two microsatellite markers FRAXAC1 and DXS548, we analysed 128 Portuguese chromosomes, including 86 controls and 42 fragile X males.

The pattern observed in the control population was very similar to that described in other studies, with C-7 being the most frequent haplotype.[28,29]

The frequency of haplotype C-7 in normal chromosomes was significantly higher than that expected from the corresponding allele frequencies. This finding may indicate the existence of linkage disequilibrium between the two markers and/or the selective advantage of this haplotype when associated with the normal *FMR1* gene as compared to C-7 fragile X chromosomes.

The higher frequencies of A-2, C-2, C-5 and D-6 haplotypes in the fragile X population suggest the existence of linkage disequilibrium between the mutant *FMR1* gene and these haplotypes. In agreement with other studies,[21–29] A-2 and D-6 were also highly prevalent in our sample (38% of patients), supporting

the contention that these two haplotypes are present on ancestral European fragile X founder chromosomes.[28,29] The presence of haplotypes C-2 and C-5 in 16 (38%) fragile X chromosomes, suggests the existence of specific founder mutations in Portuguese patients, since no similar findings have been observed in other populations.

Thus it seems that no single high-risk haplotype exists in the Portuguese population, but instead a 'protector' haplotype (C-7) could be identified accounting for 70% of the normal population and less than 3% of the fragile X chromosomes.

The greater haplotype diversity observed on fragile X chromosomes as compared with controls, which is demonstrated by the higher heterozygosity values for both FRAXAC1 and DXS548 in the former group, seems difficult to reconcile with a putative founder effect. In fact in a genetic disease, patients are expected to be less heterogeneous than the normal population. In fragile X syndrome, however, we observed that patients are more heterozygous for the linked microsatellites than are controls. Some of the low frequency haplotypes may have been formed via recombination, as seems to be the case with the A-7 fragile X associated haplotype, which could have originated by recombination between the C-7 normal and A-2 fragile X haplotypes. However, this mechanism (low-frequency haplotypes being derived from major ones by recombination) cannot explain all haplotypes associated with the disease.

A complementary explanation for the greater heterogeneity observed in the fragile X sample, could be the occurrence of a limited number of primary events (founding mutations) in a restricted number (3–6) of haplotypes, irrespective of their frequency in the control population.[24] The less frequency fragile X haplotypes (eg A-5.5, A-7 and D-7) might represent, according to this theory, more recent mutations.

Yet another mechanism which could possibly account for the greater heterogeneity found in fragile X samples has been proposed by Zhong *et al.*[28] According to this model, the *FMR1* mutation would cause an increased mutation rate of nearby microsatellite loci, either through gene conversion or localised microsatellite instability. This mechanism, however, could account for the higher than expected frequencies of A-2 and D-6 haplotypes in the fragile X population only if one assumed that this type of instability was 'directional' (non-random), favouring the generation of these (the above mentioned) haplotypes.

Alternatively, some external factor (eg a mutation at a DNA repair locus) could cause instability of this entire region.[27] However, this seems highly unlikely since we are not aware of reports on a higher incidence of fragile X syndrome in individuals, eg with Lynch syndrome and microsatellite instability due to a DNA mismatch repair deficiency. In conclusion, our results reveal significant differences in allele and haplotype frequencies (FRAXAC1 and DXS548 loci) between fragile X and normal chromosomes in Portugal, confirming the existence of specific fragile X founder chromosomes.

Acknowledgements

This work was supported by JNICT (Project PECS / C / 231 / 95).

References

1 Turner G, Webb T, Wake S, Robinson H: Prevalence of fragile X syndrome. *Am J Med Genet* 1996; **64**: 196–197.
2 Hagerman RJ: Physical and behavioral phenotype. In: Hagerman RJ, Cronister A (ed). *Fragile X Syndrome: Diagnosis, Treatment and Research*. Johns Hopkins University Press: Baltimore/London, 1996.
3 Kremer EJ, Pritchard M, Lynch M *et al*: Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CGG)n. *Science* 1991; **252**: 1711–1174.
4 Verkerk AJMH, Pieretti M, Sutcliffe JS *et al*: Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 1991; **65**: 905–914.
5 Yu S, Pritchard M, Kremer E *et al*: Fragile X genotype characterized by an unstable region of DNA. *Science* 1991; **252**: 1179–1181.
6 Fu YH, Kuhl DPA, Pizzuti M *et al*: Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* 1991; **67**: 1047–1058.
7 Snow K, Tester DJ, Kruckeberg KE, Schaid DJ, Thibodeau SN: Sequence analysis of the fragile X trinucleotide repeat: implications for the origin of the fragile X mutation. *Hum Mol Genet* 1994; **3**: 1543–1551.
8 Kunst CB, Warren ST: Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. *Cell* 1994 **77**: 853–861.
9 Pieretti M, Zhang F, Fu Y-H *et al*: Absence of expression of the FMR-1 gene in fragile-X syndrome. *Cell* 1991 **66**: 817–822.
10 Sutcliffe JS, Nelson DL, Zhang F *et al*: DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum Mol Genet* 1992; **1**: 397–400.
11 Rousseau F, Heitz D, Biahcalana V *et al*: Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. *N Engl J Med* 1991; **325**: 1673–1681.

12 Richards RI, Holman K, Friend K *et al*: Evidence of founder chromosomes in fragile X syndrome. *Nat Genet* 1992 **1**: 257–260.
13 Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acid Res* 1988; **16**: 1215.
14 Oostra BA, Jacky PB, Brown WT, Rousseau F: Guidelines for the diagnosis of fragile X syndrome. *J Med Genet* 1993; **30**: 410–413.
15 Zhong N, Dobkin C, Brown WT: A complex mutable polymorphism located within the fragile X gene. *Nat Genet* 1993; **5**: 248–253.
16 Nei M: Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 1978; **89**: 583–590.
17 Sherman SL, Morton NE, Jacobs PA, Turner G: The marker (X) syndrome: a cytogenetic and genetic analysis. *Ann Hum Genet* 1984; **48**: 21–37.
18 Sherman SL, Jacobs PA, Morton NE *et al*: Further segregation analysis of the fragile X syndrome with special reference to transmitting males. *Hum Genet* 1985; **69**: 289–299.
19 Smits A, Smeets D, Hamel B, Dreesen J, Oost B van: High prevalence of the fra(X) syndrome cannot be explained by a high mutation rate. *Am J Med Genet* 1992; **43**: 345–352.
20 Smits APT, Dreesen JCFM, Post JG: The fragile X syndrome: no evidence for any recent mutations. *J Med Genet* 1993; **30**: 94–96.
21 Hirst MC, Knight SJL, Christodoulou Z, Grewal PK, Fryns JP, Davies KE: Origins of the fragile X syndrome mutation. *J Med Genet* 1993; **30**: 647–650.
22 Jacobs PA, Bullman H, Macpherson J *et al*: Population studies of the fragile X: a molecular approach. *J Med Genet* 1993; **30**: 454–459.
23 Buyle S, Reyniers E, Vits L *et al*: Founder effect in a Belgian-Dutch fragile X population. *Hum Genet* 1993; **92**: 269–272.
24 Oudet C, Mornet E, Serre JL *et al*: Linkage disequilibrium between the fragile X mutation and two closely linked CA repeats suggests that fragile X chromosomes are derived from a small number of founder chromosomes. *Am J Hum Genet* 1993; **52**: 297–304.
25 Haataja R, Väisänen ML, Li M, Ryynänen M, Leisti J: The fragile X syndrome in Finland: demonstration of a founder effect by analysis of microsatellite haplotypes. *Hum Genet* 1994; **94**: 479–483.
26 Malmgren H, Gustavson K, Oudet C, Holmgren G, Petterson U, Dahl N: Strong founder effect for the fragile X syndrome in Sweden. *Eur J Hum Genet* 1994; **2**: 103–109.
27 Macpherson JN, Bullman H, Youings SA, Jacobs PA: Insert size and flanking haplotype in fragile X and normal populations: possible multiple origins for the fragile X mutation. *Hum Mol Genet* 1994; **3**: 399–405.
28 Zhong N, Ye L, Dobkin C, Brown WT: Fragile X founder chromosome effects: linkage disequilibrium or micro-satellite heterogeneity? *Am J Med Genet* 1994; **51**: 405–411.
29 Chiurazzi P, Genuardi M, Kozak L *et al*: Fragile X founder chromosomes in Italy: A few initial events and possible explanation for their heterogeneity. *Am J Med Genet* 1996; **64**: 209–215.
30 Morton NE, Macpherson JN: Population genetics of the fragile-X syndrome: Multiallelic model for the *FMR1* locus. *Proc Natl Acad Sci USA* 1992; **89**: 4215–4217.