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Research Article

Structure and stability upon maternal transmission of common and intermediate *FMR1* (*Fragile X Mental Retardation 1*) alleles in a sample of the Brazilian population

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Abstract

In order to investigate the stability of the *FMR1* (*Fragile X Mental Retardation 1*) alleles from the normal population, when maternally inherited, we analyzed 75 mother-to-son transmissions. Sixty-eight alleles fell within the common range with 20-40 CGG repeats, and seven alleles were intermediate, with 41-48 repeats. No change was observed either in the length or in the structure of these repeats upon transmission. Fifty-three alleles were ascertained in different families, and their size distribution was similar to those described for European and European-derived populations, with three peaks of frequency: 66% of the alleles with $(CGG)_{29}$, $(CGG)_{30}$ or $(CGG)_{31}$, 7.5% with $(CGG)_{20}$, and 5.7% with $(CGG)_{23}$. Regarding the AGG interspersion pattern, 69.8% had two AGG repeats, 20.8% had one, 5.7% had three and 3.8% had none. The most common patterns were 10+9+9 (30.2%), 9+9+9 (18.9%), 10+9 (7.5%), and 10+9+10 (7.5%). About 70% of the alleles with up to 40 repeats were linked to the DXS548/FRAXAC1 haplotype 7-3, the most commonly reported in normal populations. Four out of five intermediate alleles were in linkage with the two haplotypes most frequently associated to the *FMR1* full mutation, 2-1 and 6-4. These four alleles showed long uninterrupted CGG repeats at the 3' end. The 9+9+22, 9+9+23 and 9+9+28 alleles were linked to the haplotype 2-1, and the 9+37 allele, to the haplotype 6-4. The pattern of AGG interspersion of these alleles and the associated haplotypes were in accordance with the two main pathways toward mutation previously proposed.

Key words: FMR1 gene, CGG repeat, fragile X.

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Introduction

The fragile X syndrome is the most common X-linked mental retardation disorder affecting ~1 in 4,000 males and ~1 in 8,000-9,000 females (Crawford *et al.*, 2001). The *Fragile-X Mental Retardation 1* gene (*FMR1*) was cloned in 1991 (Oberlé *et al.*, 1991; Verkerk *et al.*, 1991; Yu *et al.*, 1991), and the vast majority of fragile-X cases are due to expansions of the polymorphic CGG trinucleotide repeat in the 5'-untranslated region of the gene. In the general population, CGG-repeat size varies from 6 to 55. In affected individuals the repeat greatly exceeds 200 and transcription silencing of *FMR1* gene occurs (Pieretti *et al.*, 1991), thus characterizing the full mutation. Alleles with repeats in the ~55-200 range, which are transcribed, but unstable, may expand to full mutations upon maternal transmission. These are known as premutations. Indeed the boundary between common and premutation alleles is not well-defined, and constitutes a "gray zone" which includes high-common and low-premutation alleles (Eichler *et al.*, 1994). These intermediate alleles are defined as those with 41~60 repeats, which may or may not be inherited in an unstable manner. (Murray *et al.*, 1997). The smallest allele known to have expanded to a full mutation had a 59 repeat, in a fragile X family (Nolin *et al.*, 2003).

Size is not the only factor implicated in allele instability. The number and position of AGG interspersions determining long uninterrupted CGG arrays at the 3' or 5' ends of the repeat, haplotype background and parental origin have been evoked as predisposing factors leading a common/intermediate allele to progress toward a mutation state (Eichler *et al.*, 1994; Kunst and Warren, 1994; Eichler *et al.*, 1996; Crawford *et al.*, 2000a; Dombrowski *et al.*, 2002; Sullivan *et al.*, 2002; Nolin *et al.*, 2003).

Herein, we report the investigation of the stability of the CGG repeat in mother-son transmissions in a random sample of common and intermediate *FMR1* alleles, analyzing repeat sizes, AGG-interspersion patterns, and linked DXS548/FRAXAC1 haplotypes.

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Subjects and Methods

Subjects

We studied 135 subjects, 59 female carriers of the *FMR1* premutation, one noncarrier female, and their 75 normal sons ascertained in the genetic counseling service of the Departamento de Biologia at the University of São Paulo, in São Paulo city, Brazil. Seventy-four mother-son pairs were selected in fragile X families through a phenotypically normal son who had inherited the normal allele from his carrier mother. In one pair, the mother carried a common and an intermediate allele. All the individuals were genotyped for the diagnosis of their carrier status and genetic counseling. The study was approved by the ethical board of the institution.

Methods

DNA was obtained from peripheral blood lymphocytes. The *FMR1* CGG-repeat size was determined by PCR using primers **c** and **f** (Fu *et al.*, 1991), according to Kenneson *et al.* (1997), with slight modifications (Mingroni-Netto *et al.*, 2002). For sequencing the CGG repeat, 100 ng of genomic DNA were amplified in a 25 µL reaction volume, with 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 50% (v/v) glycerol, 1.4 mM MgSO4, 15 pmoles **c** and **f** primers, 400 µM each dATP, dGTP, dTTP and dCTP, 1.75 U *Platinum Pfx* DNA polymerase and 3X Enhancer (Invitrogen, Carlsbad, USA). Denaturation at 94 °C for 2 min was followed by 33 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 90 s, and final extension at 72 °C for 10 min. Purification was performed with IN CONCERT™ Rapid PCR Purification System (Invitrogen, Carlsbad, USA), and 60ng of the amplified DNA were re-amplified with 3.2 pmoles of **c** or **f** primers in 6 µL Mix Big Dye (Perkin-Elmer, Foster City, USA). Denaturation at 96 °C for 10 s preceded 20 cycles of 98 °C for 10 s, and a final extension at 60 °C for 4 min. The samples were purified, precipitated and re-suspended in 12 µL Template Suppression Reagent - TSR (Perkin - Elmer, Foster City, USA). Automatic backward and forward sequencing was performed in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, USA). PCR amplifications of FRAXAC1 and DXS548 alleles were performed as previously described (Mingroni-Netto *et al.*, 1999). The allele nomenclature followed Macpherson *et al.* (1994).

Results and Discussion

The stability of the *FMR1* CGG repeat was evaluated in 75 mother-son transmissions: 46 mother-one son, 11 mother-two son (22), one mother-three son (3), and one mother-four son (4) transmissions. Fifty-three of these alleles were ascertained in different families, and their CGG repeat ranged in size from 20 to 48: 90.6% (48/53) had CGG ≤ 40, and 9.4% (5/53) were in the intermediate range (Figure 1).

The distribution of the CGG repeat sizes varies in different ethnic groups. The population of São Paulo city is ethnically heterogeneous and its composition has been in-

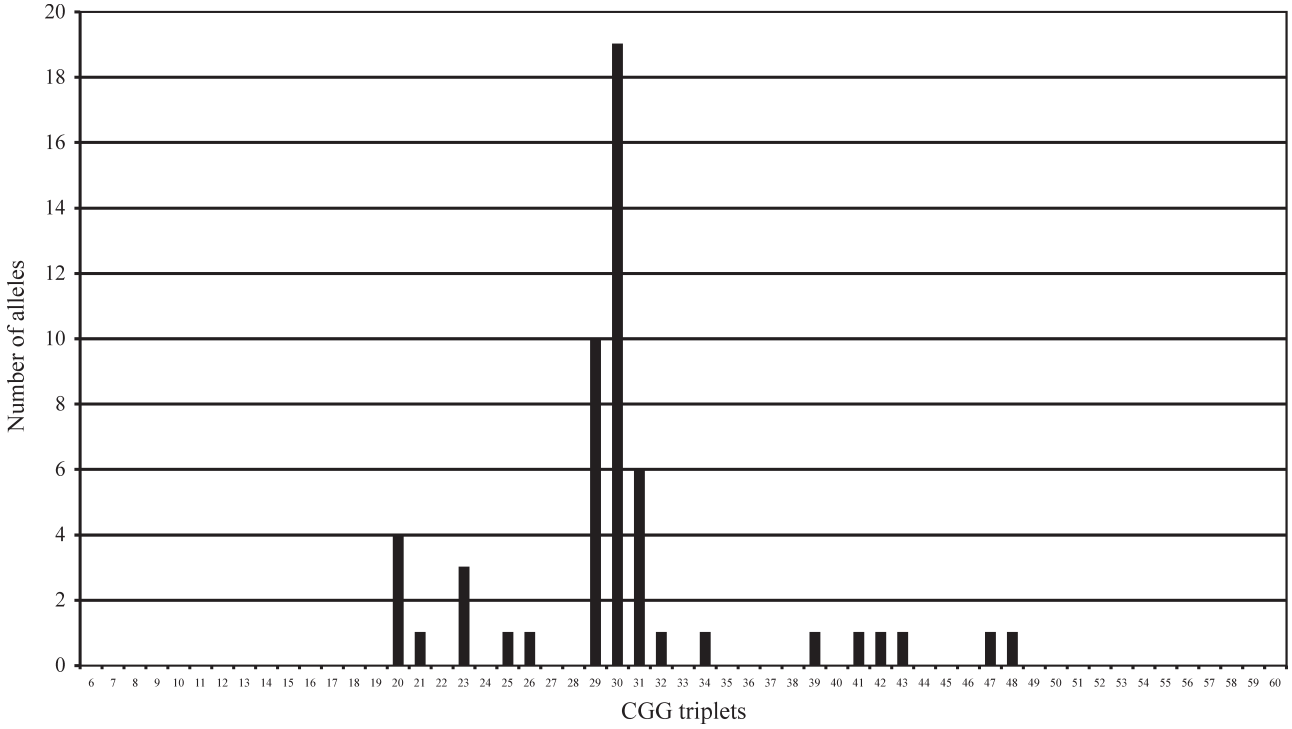

Figure 1 - *FMR1* alleles according to the length of the CGG repeat in 53 unrelated males from the population of São Paulo.

fluenced by migrations since the 16th century, with contributions from Europeans, African slaves, Asians, and in a smaller proportion of native Indians. Our sample was predominantly formed by individuals of European ancestry, and accordingly, the frequency distribution of the alleles followed those observed in European and European-derived populations, including our previous study of the São Paulo city population: a significant peak of frequency of alleles with $(CGG)_{29\text{-}31}$, a second peak around $(CGG)_{20}$, and a smaller third peak of alleles with $(CGG)_{23}$, the $(CGG)_{30}$ allele being the most frequent, (Chiurazzi *et al.*, 1996; Mingroni-Netto *et al.*, 2002). The studied alleles can therefore be considered as representing a random sample.

The stability of the repeat size upon maternal transmission

In 62.7% of the 75 transmissions (47/75) the alleles most frequent in the population were the $(CGG)_{29}$-$(CGG)_{31}$. Smaller alleles, with 20-26 triplets accounted for 24% of the transmissions (18/75), and the $(CGG)_{20}$ was the most frequent (44.4% - 8/18). Larger normal alleles, $(CGG)_{32}$ - $(CGG)_{40}$, represented 4% of the transmissions (3/75). In seven transmissions (9.3% - 7/75) the alleles were in the intermediate range. No changes were observed in the size of these alleles upon maternal transmission (95% confidential interval: 0 - 4.8%); (Table 1).

These results are in accordance with those obtained in other studies, pointing to the stability of the CGG-repeat size up to 40, when maternally inherited, with small changes observed in less than 1% of the cases (Murray *et al.*, 1997; Youings *et al.*, 2000; Sullivan *et al.*, 2002). The seven intermediate allele transmissions found in our study were all stable. Changes in intermediate range alleles, however, are more prone to occur, with a mutation rate of about 3% in maternal transmissions (Murray *et al.*, 1997; Youings *et al.*, 2000; Sullivan *et al.*, 2002). The tendency of these alleles to expand is directly related to the size of the repeat (Nolin *et al.*, 2003). Interestingly, common and intermediate alleles are less stable when transmitted through males than through females, in clear contrast with the premutated alleles (Sullivan *et al.*, 2002). Both selection against sperm with increasing repeat length and a different mutational mechanism for alleles at the common/intermediate range are possible explanations.

Table 1 - Distribution of 75 stable maternal transmissions of *FMR1* normal and intermediate alleles.

$(CGG)_n$		Number of transmissions
Maternal allele	Son allele	N = 75
20	20	8
21	21	1
23	23	6
25	25	1
26	26	2
29	29	12
30	30	22
31	31	13
32	32	1
34	34	1
39	39	1
41	41	1
42	42	1
43	43	1
47	47	2
48	48	2

CGG-repeat structure

The AGG-interspersion pattern of the CGG repeat was determined by sequencing all the 75 alleles from the normal males. The sequences of the alleles with proven common ancestry showed that no structure change had occurred within families. Among the 53 unrelated alleles, 21 patterns were identified (Table 2). In the most frequent, the sequence presented two AGG interruptions, every nine or ten CGG (69.8%; 37/53). At the 3' end, the last AGG interruption was most often followed by a nine-CGG array. Among the most frequent allele sizes, $(CGG)_{30}$ had characteristically (16/19) the 10+9+9 structure (+ standing for AGG), $(CGG)_{29,}$ the structure 9+9+9 (10/10), and $(CGG)_{31}$, 10+9+10 (4/6). All four $(CGG)_{20}$ alleles were 10+9. Alleles with three AGG interspersions represented 5.7% (3/53) of the sample - one $(CGG)_{30}$ - 10+7+1+9, one $(CGG)_{39}$ - 9+9+9+9 and one intermediate allele $(CGG)_{41}$ - 9+10+10+9. Alleles with pure CGG triplets accounted for 3.8% (2/53) of the sequences - one $(CGG)_{23}$ and one $(CGG)_{26}$, all alleles ≤ 30. Eleven alleles (20.8%) had one AGG interspersion - four $(CGG)_{20}$, one $(CGG)_{21}$, two $(CGG)_{23}$, one $(CGG)_{25}$, one $(CGG)_{30}$, one $(CGG)_{34}$ and one $(CGG)_{47}$, therefore, only two alleles ≥ 30. Four of these alleles had more than 10 CGG at the 5' end, before the first AGG interspersion: two $(CGG)_{23}$ - 13+9, one $(CGG)_{25}$ - 15+9 and one $(CGG)_{30}$ - 20+9. Two alleles had 24 and 37 CGG uninterrupted arrays at the 3' end: $(CGG)_{34}$ - 9+24 and $(CGG)_{47}$ - 9+37.

Eichler *et al.* (1994) and Eichler *et al.* (1995) were the first to report that the majority of CGG repeats presented two AGG interspersions, and that large uninterrupted CGG sequences were at the 3' end, pointing to polarity in instability. The loss of the most 3' AGG or its conversion to CGG was proposed as predisposing to instability. In African-Americans, however, Crawford *et al.* (2000b) identified a large uninterrupted CGG repeat at the 5' end as a probable predisposing factor to instability, thus disclosing a population-dependent pathway to mutation.

Table 2 - Distribution of 53 *FMR1* common and intermediate alleles, according to the length of the repeat, AGG interspersion patterns, and linked DXS542/FRAXAC1 haplotypes.

AGG interruption pattern*	DXS548/FRAXAC1 haplotype	Number of CGG triplets																Total of alleles
		20	21	23	25	26	29	30	31	32	34	39	41	42	43	47	48	
10+9	7-3	4																4
10+10	7-2		1															1
23	7-3			1														1
13+9	7-2			1														1
	7-3			1														1
15+9	7-3				1													1
26	7-3					1												1
9+9+9	7-3						3											3
	7-2						2											2
	2-4						2											2
	7-4						1											1
	7-6						1											1
	5-4						1											1
10+9+9	7-3							14										14
	6-3							1										1
	8-3							1										1
20+9	7-3							1										1
9+9+10	5-2							1										1
10+7+1+9	7-3							1										1
10+9+10	7-3								4									4
10+10+9	7-1								2									2
9+9+12	7-3									1								1
9+24	7-3										1							1
9+9+9+9	2-1											1						1
9+10+10+9	7-3												1					1
9+9+22	2-1													1				1
9+9+23	2-1														1			1
9+37	6-4															1		1
9+9+28	2-1																1	1
Total of alleles		4	1	3	1	1	10	19	6	1	1	1	1	1	1	1	1	53

*Numbers = CGG triplets; + = AGG.

AGG-interspersion patterns and linked DXS548/FRAXAC1 haplotypes

The DXS548 and FRAXAC1 microsatellite loci are tightly linked to the *FMR1* gene (Richards *et al.*, 1991; Riggins *et al.*, 1992). The 7-3 (194 bp/154 bp) haplotype was the most frequent in our sample (34/53; 64.1%), as observed on 50% - 70% of the chromosomes in European and European-derived populations (Chiurazzi *et al.*, 1996; Peixoto *et al.*, 1998; Mingroni-Netto *et al.*, 1999; Crawford *et al.*, 2000a). Haplotypes frequently associated to fragile X chromosomes in these populations (2-1, 204 bp/158 bp, and 6-4, 196 bp/152bp) represented 9.4% of our sample. We did not observe the haplotypes 4-4 (200 bp/152 bp) or 3-4 (202 bp/152 bp), reported to be frequent on fragile X chromosomes of African-Americans (Crawford *et al.*, 2000a).

We analyzed the 53 unrelated alleles regarding the pattern of AGG-interspersion and the linked DXS548/FRAXAC1 haplotype (Table 2). Around 47% (25/53) of the chromosomes had the 7-3 haplotype associated with the commonest normal alleles: $(CGG)_{30}$ - 10+9+9; $(CGG)_{31}$ - 10+9+10; $(CGG)_{29}$ - 9+9+9, and $(CGG)_{20}$ - 10+9. The most frequent association was the pattern 10+9+9 and the 7-3 haplotype, on more than 20% of the chromosomes, as already reported (Eichler *et al.*, 1996; Gunter *et al.*, 1998; Ennis *et al.*, 2001). The pattern 9+9+9 was linked to the highest number of different haplotypes (six), and it was associated with the 7-3 haplotype only on

5.6%(3/53) of the chromosomes. Indeed, the observation that in African-Americans the 9+9+9 repeat was associated with the highest haplotype diversity had already suggested that this is probably the ancestral CGG repeat structure (Crawford *et al.*, 2000b).

Among the 48 alleles with up to 40 triplets, the fragile X haplotype 2-1 was observed only once linked to a $(CGG)_{39}$ - 9+9+9+9, and the haplotype 6-4 was not found. Eight of these 48 common alleles had uninterrupted CGG sequences with more than 10 triplets, and seven (87.5%) were linked to the commonest 7-3 haplotype: two pure CGG sequence alleles -$(CGG)_{23}$ and $(CGG)_{26}$; one $(CGG)_{23}$ - 13+9; one $(CGG)_{25}$ - 15+9; one $(CGG)_{30}$ - 20+9; one $(CGG)_{32}$ - 9+9+12, and one $(CGG)_{34}$ - 9+24. The other allele, $(CGG)_{23}$ - 13+9, was linked to the haplotype 7-2 (194 bp/156 bp). None of these haplotypes had been significantly represented on reported fragile X chromosomes.

On the other hand, four out of the five intermediate alleles were linked to the haplotypes most frequently associated with fragile X chromosomes. They presented large uninterrupted sequences at the 3' end: three alleles $(CGG)_{42}$, $(CGG)_{43}$ and $(CGG)_{48}$, associated with the 2-1 haplotype, showed the pattern 9+9+X, with X = 22, 23 and 28 CGG repeats. The allele $(CGG)_{47}$ had a 9+37 pattern, and was associated with the 6-4 haplotype. The remaining intermediate allele, $(CGG)_{41}$, had three AGG interspersions (9+10+10+9), and was linked to the 7-3 haplotype.

Kunst and Warren (1994) first reported that alleles with long uninterrupted CGG repeats at the 3' end were in linkage disequilibrium with the haplotypes most frequently associated with the fragile X chromosomes, thus constituting the pool of alleles from which the expanded mutated alleles were derived. Based on the structure of the repeats, and the linked haplotypes, Eichler *et al.* (1996) proposed two main mutational pathways in the origin of the fragile X mutation. In ancestral CGG repeats with an asymmetrical structure, associated with the 6-4 haplotype, the recurrent loss of AGG interruptions would lead to a relatively rapid progress to the mutation. The alleles linked to the 2-1 haplotype, which maintained two AGG interspersions, would generate larger alleles by gradual increases in CGG repeats distal to the most 3' AGG. More recently, a study of the English population revealed that alleles associated with the 6-4 haplotype showed the structure 9+X+Y (X > 9CGG triplets and Y, any sequence size) or 9+X; in turn, alleles on 2-1 haplotypes had patterns 9+9+X (Ennis *et al.*, 2001). Again in accordance with the two-mutation-pathway hypothesis, the intermediate alleles linked to 2-1 haplotype in our sample had the 9+9+X pattern, and the 9+37 structure was associated with the 6-4 haplotype.

Our data confirms that common FMR1 alleles (CGG $\leq$ 40) are quite stable upon maternal transmission. The most frequent DXS548/FRAXAC1 haplotypes on fragile X chromosomes were not found to be associated with the common alleles with structures that would favor expansions, such as pure CGG sequences or 5' or 3' uninterrupted CGG $\geq$ 20. On the other hand, uninterrupted CGG sequences of similar sizes on alleles with more than 40 triplets were always associated with fragile X haplotypes. Whether the linked haplotype or other cis-acting sequence somehow influence or not the tendency of these alleles to expand remains unclear.

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