Haplotype and AGG Interspersion Analysis of FMR1 Alleles in a Croatian Population: No Founder Effect Detected in Patients with Fragile X Syndrome

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Abstract Several studies have suggested that fragile X syndrome (FRAXA), the most common inherited form of mental retardation, originated from a limited number of founder chromosomes. The aim of this study is to assess the genetic origin of fragile X syndrome in a Croatian population. We performed a haplotype analysis of the polymorphic loci *DXS548* and *FRAXAC1* in 18 unrelated fragile X and 56 control chromosomes. The AGG interspersion pattern of the *FMR1* CGG repeat region was analyzed by sequencing. This is the first report on haplotype and AGG interspersion analysis of the fragile X syndrome gene in a Croatian population—the only eastern European population of Slavic origin analyzed so far. Our findings are intriguing, because they show a distinct distribution of the *DXS548* and *FRAXAC1* alleles in our fragile X population compared to other European fragile X populations. The *DXS548*/*FRAXAC1* haplotype 194/154 (7-3), which is common among normal populations, was found to be the most frequent haplotype in our fragile X population as well. The AGG interspersion analysis indicated that AGG loss rather than haplotype may determine *FMR1* allele instability. Our results suggest that no common ancestral X chromosome is associated with fragile X syndrome in the Croatian population studied. Further analysis of the origin of fragile X chromosomes among other Slavic populations will be necessary to better define their eastern European distribution.

Fragile X syndrome (FRAXA, Xq27.3) is the most common inherited form of mental retardation. It is generally caused by an expansion of the CGG repeat region within the *FMR1* gene. Normal alleles contain about 5–50 CGG repeats, whereas carriers of the disease have about 50–200 repeats (termed premutations) and affected individuals have more than 200 CGG repeats (termed full mutations). The CGG repeats of normal alleles are interrupted by an AGG triplet every 9 or 10 CGG repeats, and the loss of AGG repeats has been proposed to destabilize the repeat, making it prone to expansion (Kunst and Warren 1994; Larsen et al. 2000).

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Polymorphic markers near the gene have shown linkage disequilibrium with fragile X chromosomes, suggesting that a pool of founder chromosomes account for fragile X syndrome (Chiurazzi et al. 1996b; Kunst and Warren 1994). In Croatia we have genetically diagnosed fragile X syndrome since 1996 and have identified 24 fragile X families (FRAXA) and 2 families with the more rare form of the fragile X chromosome (FRAXE) so far. The aim of this study is to assess the genetic origin of fragile X syndrome in a Croatian population. We performed haplotype analysis of microsatellites *DXS548* and *FRAXAC1* between a control group and a fragile X group and determined the AGG interspersion pattern within the CGG sequence of the *FMR1* normal alleles.

Materials and Methods

DNA Sample. Eighteen DNA samples from unrelated males with fragile X syndrome and 56 DNA samples from unrelated male control subjects were included in this study. These samples were obtained through the routine genetic diagnosis of fragile X syndrome.

Haplotype Analysis. Alleles of the two polymorphic loci, *DXS548* and *FRAXAC1*, which lie 150 kb and 7 kb proximal to the *FMR1* gene, were analyzed by duplex PCR (Pekarik et al. 1999). Consistency of allele denomination was ensured by typing control DNAs kindly provided by I. Arrieta (Facultad de Ciencias, Bilbao, Spain) and J. N. Macpherson (Salisbury Hospital, Salisbury, England).

AGG Interspersion Analysis. The CGG triplets in the *FMR1* gene were first amplified (Hecimovic et al. 1997). After purification, the forward and reverse strands were then sequenced using BigDye Terminator v1.1 (Applied Biosystems, Foster City, California) in an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Statistical Methods. The Fisher exact test was used to test the significance of the differences between frequencies of alleles and haplotypes in the fragile X and control groups. We used Statistica for Windows, version 7, to perform the test. Marker heterozygosity was calculated with the formula

$$\text{Het} = 1 - \sum q^2, \tag{1}$$

where q is the frequency of each individual allele at the *DXS548* or *FRAXAC1* locus.

Results and Discussion

Allele frequencies at the *DXS548* and *FRAXAC1* loci are shown in Table 1. In agreement with previous reports, we observed an increase in heterozygosity

Table 1. Distribution and Frequency of the *DXS548* and *FRAXAC1* Alleles in the Fragile X and Control Groups[a]

	DXS548	*FRAXAC1 Allele*						
Group	*Allele*	*158 (1)*	*156 (2)*	*154 (3)*	*152 (4)*	*150 (5)*	*Sum*	*f (%)*
Fragile X group	206 (1)							
	204 (2)							
	202 (3)		1	2			3	16.67
	200 (4)			1			1	5.55
	198 (5)				1		1	5.55
	196 (6)				3		3	16.67
	194 (7)			8	2		10	55.56
	Sum		1	11	6		18	
	f (%)		5.55	61.11	33.33			
Control subjects	206 (1)							
	204 (2)		1				1	1.78
	202 (3)	1	1	1	1		4	7.14
	200 (4)			1			1	1.78
	198 (5)							
	196 (6)			3	2		5	8.93
	194 (7)	1	1	37	5	1	45	80.36
	Sum	2	3	42	8	1	56	
	f (%)	3.57	5.36	75.00	14.29	1.78		

Sum, subjects with a specific *DXS548* or *FRAXAC1* allele.

f (%), frequency.

a. The nomenclature for CA repeats of Pekarik et al. (1999) and Eichler et al. (1996) were used (numbers in parentheses). Numbers correspond to individuals with a particular haplotype.

in the fragile X group (*DXS548*, 63%; *FRAXAC1*, 51.27%) relative to the control subjects (*DXS548*, 34.1%; *FRAXAC1*, 41.26%). However, no statistically significant differences in *DXS548* and *FRAXAC1* allele frequencies between our control and fragile X groups were detected. Interestingly, in contrast to other European populations, *DXS548* allele 194 (allele 7) was the most frequent in both the control and fragile X groups, and neither allele *DXS548* 204 (allele 2) or *FRAXAC1* 158 (allele 1) was detected in our fragile X chromosomes (Buyle et al. 1993; Chiurazzi et al. 1996a; Malmgren et al. 1994; Pekarik et al. 1999; Peñagarikano et al. 2004).

Among 74 X chromosomes analyzed, 14 different haplotypes were detected (Figure 1): 13 haplotypes in the control group and 7 in the fragile X group. The most common *DXS548*/*FRAXAC1* haplotype among normal chromosomes was 194/154 (7-3, 66.07%), which is consistent with previous findings. Intriguingly, the same haplotype was also the most common among fragile X chromosomes (44.44%). In contrast to our findings, haplotypes 196/152 (6-4) and 204/158 (2-1) were the most abundant among fragile X chromosomes in other European populations (Buyle et al. 1993; Chiurazzi et al. 1996a; Larsen et al. 2000; Macpherson et al. 1994; Malmgren et al. 1994; Pekarik et al. 1999; Peñagarikano et al. 2004). Although the frequency of haplotype 196/152 (6-4) was increased in our fragile

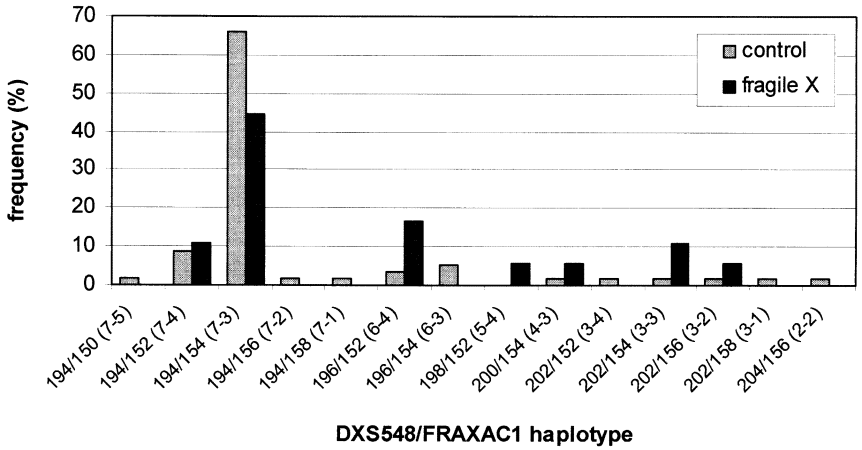

Figure 1. Distribution and frequency of *DXS548/FRAXAC1* haplotypes in the control group and the fragile X group. In contrast to other reports, *DXS548/FRAXAC1* haplotype 194/154 (7-3) is the most frequent among our fragile X chromosomes. Although the frequency of haplotype 196/152 (6-4) was increased in our fragile X group compared to control subjects (16.67% vs. 3.57%), this difference was not statistically significant ($p = 0.0542$, $\lambda^2 = 3.71$, df $=$ 1). The nomenclatures for CA repeats of Pekarik et al. (1999) and Eichler et al. (1996) were used (numbers in parentheses).

X group compared to the control group (16.67% vs. 3.57%), this difference was not statistically significant ($p = 0.0542$, $\lambda^2 = 3.71$, df $= 1$). In addition, we did not detect haplotype 204/158 (2-1) in our fragile X chromosomes. A similar frequency of haplotype 196/152 (6-4) in fragile X chromosomes from our fragile X group (16.67%), Italians (16.8%; Chiurazzi et al. 1996a), and Czechs (16.2%; Pekarik et al. 1999) probably reflects the southeastern European component and may be compatible with the location of Croatia in Europe. The same may be true for haplotype 194/154 (7-3), which is also frequent on fragile X chromosomes in the Mediterranean area (20.8% in Italy; Chiurazzi et al. 1996a) and is the most frequent in our fragile X group. However, our data may imply that the fragile X mutation in the Croatian population occurs on a background of a common haplotype 194/154 (7-3). In contrast to other European populations, we did not observe linkage disequilibrium between *DXS548/FRAXAC1* haplotypes and control or fragile X alleles; however, we could not exclude linkage disequilibrium because of the small sample size. Recently, no founder haplotype among fragile X patients in Taiwan (Limprasert et al. 2001) or in a specific ethnic population of Ashkenazi Jews (Pesso et al. 1997) has been reported.

Analysis of the AGG interspersion pattern within the CGG repeat region in the *FMR1* gene revealed 26 different CGG repeat structures of 56 control chromosomes. Table 2 summarizes the distribution of 26 different AGG interspersion pat-

Table 2. Distribution of AGG Interspersion Patterns and *DXS548*/*FRAXAC1* Haplotypes Among *FMR1* CGG Normal Alleles

CGG Allele	*Haplotype*	*AGG Pattern*	*N*
45	202–156 (3–2)	9 + 30 + 4	1
41	194–154 (7–3)	10 + 9 + 10 + 9	
		10 + 9 + 20	1
40	194–154 (7–3)	10 + 9 + 19	1
39	202–158 (3–1)	9 + 9 + 9 + 9	1
37	194–154 (7–3)	9 + 9 + 7 + 9	2
36	194–152 (7–4)	8 + 27	1
	194–154 (7–3)	10 + 25	1
32	194–152 (7–4)	9 + 12 + 9	1
31	194–154 (7–3)	10 + 9 + 10	6
	196–154 (6–3)	10 + 9 + 10	1
	202–152 (3–4)	9 + 11 + 9	1
30	194–150 (7–5)	10 + 9 + 9	1
	194–154 (7–3)	10 + 9 + 9	10
	194–156 (7–2)	10 + 9 + 9	1
	194–158 (7–1)	10 + 9 + 9	1
	196–154 (6–3)	10 + 9 + 9	1
29	194–152 (7–4)	9 + 9 + 9	1
		19 + 9	1
	194–154 (7–3)	9 + 9 + 9	5
		10 + 9 + 8	1
	204–156 (2–2)	9 + 9 + 9	1
27	194–154 (7–3)	10 + 9 + 6	1
		10 + 16	1
26	196–152 (6–4)	16 + 9	1
24	202–154 (3–3)	13 + 10	1
23	194–152 (7–4)	13 + 9	1
	196–152 (6–4)	9 + 13	1
	200–154 (4–3)	13 + 9	1
22	194–154 (7–3)	9 + 12	1
		10 + 11	1
		11 + 10	1
		12 + 9	1
20	194–154 (7–3)	10 + 9	2
	196–154 (6–3)	10 + 9	1

terns of *FMR1* CGG repeat sizes and *DXS548*/*FRAXAC1* haplotypes. Most AGG repeat interspersion patterns contained either one (28.6%) or two AGG repeats (62.5%). There were no normal alleles without AGG. The most common CGG repeat structures were 10 + 9 + 9 (25%), 9 + 9 + 9 (12.5%), and 10 + 9 + 10 (12.5%) (Eichler et al. 1996). All three structures were concentrated primarily on the 194/154 (7-3) haplotype (17/24, 70.83%).

In Caucasian populations it has been hypothesized that intermediate alleles with at least 24 pure CGG repeats at the 3′ end of the repeat sequence are prone

to repeat instability and expansion (Eichler et al. 1996; Kunst and Warren 1994; Zhong et al. 1995). Of the 56 control chromosomes, only 3 alleles were identified to have at least 24 pure CGG repeats, and all three were the gray-zone normal alleles ($35 \leq \text{CGG} < 60$). Two of these alleles had pure CGG structure at the 3′ end of the repeat sequence ($8 + 27$ and $10 + 25$) with only one AGG interruption, whereas in one allele the loss of AGG was detected in the middle of the *FMR1* CGG sequence ($9 + 30 + 4$). In four out of five (80%) of the gray-zone alleles with at least 15 pure CGG repeats, the loss of AGG occurred at the 3′ end and in three out of five (60%) gray-zone alleles haplotype 194/154 (7-3) was observed (Arrieta et al. 2003). Although the number of the gray-zone alleles was small (10/56), our results indicate that the loss of AGG interruptions at the 3′ end in these alleles may account for the occurrence of the fragile X mutation among haplotypes commonly found in the normal population.

This study is the first report on fragile X genotyping of an eastern European population of Slavic origin. The two-marker analysis did not reveal a distinct haplotype prevalent in our fragile X group, suggesting that no common ancestral X chromosome is associated with the fragile X syndrome in the Croatian population studied. These findings are in contrast to other reports on founder effects associated with fragile X syndrome in other European populations and in the only Slavic population analyzed so far, the Czech Republic. AGG interspersion analysis revealed that AGG loss rather than haplotype may determine *FMR1* allele instability in our population. More studies on the origin of fragile X syndrome in other Slavic populations will be necessary to define the eastern European distribution of fragile X chromosomes.

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