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Clinical Report

Haplotype Analysis at the FRAXA Locus in an Indian Population

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The FRAXA locus is flanked by three polymorphic STR markers DXS548, FRAXAC1, and FRAXAC2. Allele frequencies of these markers were determined on a population representing the eastern part of India comprising of 69 normal controls and 69 unrelated subjects with mental retardation, among whom 21 were fragile X patients. These frequencies were compared with published data on other Indian population and the major populations of the world. The allele and haplotype distribution of the studied population were significantly different in some respects from the major populations of the world. The increase of heterozygosities in fragile X samples (DXS548 67.5%, FRAXAC1 63.5%, FRAXAC2 68.5%) relative to the controls (DXS548 63.3%, FRAXAC1 51.0%, FRAXAC2 67.2%) suggests a multimodal distribution of fragile X associated alleles. Haplotype analyses with DXS548 and FRAXAC1 markers revealed that haplotype distribution in the normal controls and fragile X groups were significantly different, suggesting a weak founder effect. © 2008 Wiley-Liss, Inc.

Key words: STR; *FMR1*; haplotypes; Indian population

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INTRODUCTION

Fragile X syndrome, the most common form of inherited mental retardation with an incidence of 1 in 4,000 males and 1 in 8,000 females [Warren and Nelson, 1994; Warren and Sherman, 2000] stems from reduction or loss of the FMR1 protein. The gene concerned, *FMR1* (fragile X mental retardation) is a highly conserved one consisting of 17 exons spanning over 38 kb. The disease is caused mainly by the expansion of a polymorphic $(CGG)_n$ repeat located in the 5′-untranslated region of the *FMR1* gene [Verkerk et al., 1991]. The normal repeat size ranges from 7 to ~60 with ~30 repeats on the most frequent alleles. In the clinically affected individuals, there is a massive CGG expansion (>200 repeats) called full mutation. Alleles containing CGG repeats in the range of 60–200 are called premutations and do not associate with mental retardation. Both kinds of alleles are meiotically unstable and mosaicism in individuals suggests mitotic instability as well. Extensive polymorphism of the CGG repeat has been observed in the normal individuals, varying from 6 to 54 in the Caucasians [Fu et al., 1991] and 8 to 50 in the Indian population [Baskaran et al., 1998; Saha et al., 2001; Sharma et al., 2001]. Reports on this disorder from the Indian population, employing DNA diagnostics method revealed a frequency of 7–8% from the various parts of the country [Baskaran et al., 1998; Saha et al., 2001; Sharma et al., 2001].

The genetic localization of the FRAXA fragile X site at Xq 27.3 associated with fragile X syndrome was reported and the position of the fragile site within the multipoint linkage map was determined using two polymorphic microsatellite AC repeat markers FRAXAC1 and FRAXAC2 [Richards et al., 1991]. These markers were located within 10 kb and on either side of $(CGG)_n$ repeat of the *FMR1* gene and provided an accurate means of diagnosis of the fragile X genotype

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in families by rapid PCR analysis. Another highly polymorphic dinucleotide repeat DXS548 represented by CA repeats and located 150 kb proximal to the *FMR1* gene was characterized [Riggins et al., 1992]. This marker also proved to be useful in determining the origin of fragile X mutations. With the discovery of these polymorphic markers, it became possible to mark genetic haplotypes on the fragile X chromosome. Linkage disequilibrium mapping was done between the three polymorphic AC markers and the CGG repeat. This mapping is based upon the expectation that if all mutant chromosomes descended from a common ancestral mutation, then these markers in close proximity to the mutant gene, will show a common haplotype reflecting that of the original ancestral chromosome. A number of studies have found significantly different haplotype frequencies in samples of fragile X and of normal chromosomes [Richards et al., 1992; Buyle et al., 1993; Hirst et al., 1993; Oudet et al., 1993a,b]. It has been observed that CGG repeats at the high end of normal range show a similar distribution of flanking haplotypes as normal X chromosomes [Richards et al., 1992; Jacobs et al., 1993]. This suggests the possibility that fragile X mutations are derived from a limited number of initial expansions representing a "founder effect." Fragile X founder effects have been established in various populations of the world British [Macpherson et al., 1994], Finnish [Zhong et al., 1996], Japanese [Richards et al., 1994], Italian [Chiurazzi et al., 1996a], Argentineans [Bonaventure et al., 1998], Chileans [Jara et al., 1998], and Chinese [Zhong et al., 1999] where there appears to be a common genetic background on which FMR1 expressions have occurred. A study on the Indian population mainly from the northern part of the country [Sharma et al., 2003] revealed a high haplotype diversity and a weak founder effect possibly arising due to complex demographic heterogeneity of the population. There are some fragile X chromosomes, which carry other haplotypes suggesting that the mutations have arisen independently on other genetic backgrounds. A model was proposed where fragile X mutations were postulated to occur as a multistep process [Morton and Macpherson, 1992]. This model suggested that alleles over 50 copies of CGG arise from normal alleles but these are non-phenotypic and stable for many generations. This longevity would allow the required time for establishment of an ancestral haplotype resulting in the observed linkage disequilibrium.

The present study has been conducted on individuals living in and around Calcutta and they represent a part of eastern India. We report on the polymorphism of the three markers DXS548, FRAXAC1, and FRAXAC2 and the haplotype analysis using DXS548 and FRAXAC1 markers on this population. A specific association of some haplotypes with normal control or fragile X group was detected. Allele and haplotype distribution in the normal controls and fragile X groups were significantly different, suggesting weak founder effect in the population studied.

MATERIALS AND METHODS

Subjects

The control population included in this study consisted of 69 (50 males and 19 females) individuals. Exclusion criteria were the absence of CGG expansion and the distribution of CGG repeats in this group closely resembled to that of control samples used in previous studies [Fu et al., 1991]. The controls included healthy volunteers and the fathers of the patients with unrelated mental retardation (UMR). The group of idiopathic mentally retarded subjects (n = 48; 33males and 15 females) consisted of patients with UMR. The clinicians sent them to us from the institutes mentioned below.

The institutes are:

(i) Department of Pathology, Post Graduate Institute of Medical Research, Calcutta.
(ii) Bangur Institute of Neurology, Calcutta.
(iii) Bodhipeet, a center for the mentally retarded, Calcutta.
(iv) Manovikas Kendra, Calcutta.
(v) Institute of Child Health, Calcutta.

In addition to the cases of UMR, 19 males and 2 females were detected fragile X positive.

The chromosomes of the subjects were screened for the DXS548, FRAXAC1, and FRAXAC2 marker alleles and haplotypes were constructed on that basis. Haplotypes of females could be constructed only when one of the loci was homozygous, or data of their sons were also available. Not all persons could be successfully typed at each loci. Written informed consent was obtained from the normal individuals and the patients or their guardians for including them in this study.

DNA Analyses

Genomic DNA was extracted from leucocytes using the protocol of Miller et al. [1988]. Detection of CGG alleles in the *FMR1* gene by radioactive PCR–PAGE method using primers C and 571R and confirmation of fragile X positive individuals by Southern blot analysis were done as described by Saha et al. [2001].

The DXS548 AC repeat polymorphism was amplified using forward and reverse primers DXSF and DXSR in amplification conditions described by Riggins et al. [1992]. FRAXAC1 and FRAXAC2 polymorphisms were detected as described in Richards et al. [1991]. The PCR products were resolved on 6%

denaturing polyacrylamide sequencing gel and sized relative to alleles, which has been sequenced using ABI 3130 genetic analyzer. The alleles were named according to Chiurazzi et al. [1999].

Statistical Analysis

For statistical analysis, expected marker heterozygosity was calculated using the formula $1-\sum q^2$ where "q" is the frequency of each individual allele at FRAXAC1, FRAXAC2, or DXS548 locus. The Fisher–Freeman exact test was employed to test significance of differences between allele frequencies and haplotypes in fragile X patients and controls (using Statistica 7.0 software).

RESULTS

Allele Polymorphism at DXS548, FRAXAC1, and FRAXAC2 Loci

The distribution of DXS548, FRAXAC1, and FRAXAC2 alleles have been shown in Table I in cases of normal controls, persons with UMR, and fragile X patients. The frequency in percent is given in parenthesis.

DXS548 alleles were named by base pair marking as described by Riggins et al. [1992]. FRAXAC1 and FRAXAC2 alleles were also represented by base pairs [Richards et al., 1991]. Number of CA repeats corresponding to the size of allele in base pairs has been given in parenthesis. This is followed by the nomenclature suggested by Chiurazzi et al. [1999].

Heterozygosity of DXS548 was found to be 63.3% in normals, 62.8% in UMR, and increased to 67.5% in the fragile X group. In FRAXAC1, heterozygosities observed for the control population was 51%, for the group of unrelated mentally retarded it was 60.21%, and for fragile X patients it was 62.8%. In FRAXAC2, respective values were 67.2, 78.5, and 68.5%.

The DXS548 allele distribution in the control and fragile X groups were found to be significantly different ($P=0.0032$). The allele distribution of FRAXAC1 in the control and fragile X groups are not significantly different ($P=0.08$). The allele distributions at FRAXAC2 locus in the controls and fragile X groups are significantly different ($P=0.002$).

Haplotype Analysis

We analyzed haplotypes using the markers DXS548 and FRAXAC1. We did not use the other marker FRAXAC2 because this marker contains the complex microsatellite structure $(GT)_x$-C-$(TA)_y$-$(T)_z$ and can create complications in the determination of correct haplotypes. The haplotype distribution is shown in Table II. For representation of haplotypes, DXS548 alleles have been named as per Richards et al. [1991], that is, 9 (190), 8 (192), 7 (194), 6 (196), 5 (198), 4 (200), 2 (204) and FRAXAC1 alleles have been named as per Riggins et al., that is, E (150), D (152), C (154), B (156), and A (158). Statistical analysis showed that the haplotypes distribution between the controls and fragile X groups is

TABLE I. Allele Frequency of DXS548, FRRAXAC1, and FRAXAC2 Alleles

Allele (bp) (CA repeats)	Normals (%)	Unrelated mental retardation (%)	Fragile X patients (%)
DXS548			
190 (18)/9	—	2 (3.63)	5 (23.8)
192 (19)/8	24 (41.37)	29 (52.72)[a]	9 (42.9)[a]
194 (20)/7	25 (43.1)[a]	16 (29.10)	6 (28.57)
196 (21)/6	5 (8.62)	2 (3.63)	—
198 (22)/5	1 (1.72)	1 (1.81)	—
202 (24)/3	3 (5.17)	4 (7.27)	—
204 (25)/2	—	1 (1.81)	1 (4.76)
Total	58	55	21
Heterozygosity	63.25%	62.8%	67.5%
FRAXAC1			
150 (17)F	1 (2.08)	4 (6.34)	—
152 (18)E	15 (31.25)	32 (50.79)[a]	9 (42.85)[a]
154 (19)D	30 (62.5)[a]	23 (36.50)	8 (38.1)
156 (20)C	—	1 (1.58)	—
158 (21)B	2 (4.16)	3 (4.76)	4 (19.05)
Total	48	63	21
Heterozygosity	51%	60.21%	63.5%
FRAXAC2			
151 (5+)	3 (3.40)	2 (9.52)	—
152 (5)	—	2 (9.52)	1 (4.76)
153 (4+)	37 (42.04)[a]	5 (23.8)	5 (23.8)
154 (4)	22 (25)	6 (28.6)[a]	10 (47.61)[a]
155 (3+)	26 (29.54)	5 (23.8)	3 (14.28)
156 (3)	—	1 (4.76)	2 (9.52)
Total	88	21	21
Heterozygosity	67.22%	78.5%	68.5%

[a]Denotes the most frequent allele.

TABLE II. Distribution of DXS548–FRAXAC1 Haplotypes in the Studied Population Comprising of Normal Individuals, Fragile X Patients, Normal Individuals With More Than 30 Repeats and Permutation Females

Haplotypes	Normal	Fragile X patients	Normal (≥30)
(9E) 190–152	—	4 (19.0)	—
(9C) 190–154	—	1 (4.8)	—
(8D) 192–152	8 (17.8)	2 (9.5)	—
(8C) 192–154	3 (6.7)	5 (23.8)[a]	1 (8.3)
(8A) 192–158	—	2 (9.5)	—
(7E) 194–150	1 (2.2)	—	—
(7D) 194–152	5 (11.1)	3 (14.3)	1 (8.3)
(7C) 194–154	20 (44.4)[a]	2 (9.5)	7 (58.3)[a]
(7A) 194–158	1 (2.2)	1 (4.8)	—
(6C) 196–154	3 (6.7)	—	—
(5C) 198–154	1 (2.2)	—	1 (8.3)
(3C) 202–154	2 (4.4)	—	2 (16.7)
(3A) 202–158	1 (2.2)	—	—
(2A) 204–158	—	1 (4.8)	—
Total	45	21	12

[a]denotes the most frequent allele.

significantly different ($P = 0.006$). However, haplotypes shared by chromosomes bearing >30 repeats are more like the controls ($P = 0.81$).

DISCUSSION

The increase of heterozygosities in fragile X samples (DXS548 67.5%, FRAXAC1 62.8%, FRAXAC2 68.5%) relative to the controls (DXS548 63.3%, FRAXAC1 51%, FRAXAC2 67.2%) have been observed in all the three tested marker loci. The increase of heterozygosity in fragile X patients have been observed in almost every population studied [Zhong et al., 1994; Bonaventure et al., 1998; Pekarik et al., 1999; Limprasert et al., 2001] and this reflects multimodal distribution of the fragile X associated alleles and the presence of one dominant allele in the controls. We also found that heterozygosity of all markers was higher in fragile X samples in comparison to the normal controls.

DXS548 is characterized by a unimodal distribution with a high frequency of 80–90% of the 194 allele in many populations like the Caucasians, Chinese, Japanese, and the Thais. The heterozygosity of the control population (63.3%) studied by us is higher in comparison to the Chinese (33%), Thai (16.5%), and Caucasians (44%), which reflects the heterogeneity of the studied population. A study on an Indian population from the northern part of India [Sharma et al., 2003] also reveals a lower prevalence of the 194 allele, a situation similar to that observed among the populations of Cameroon and blacks in USA [Chiurazzi et al., 1996b and Crawford et al., 2000]. The modal allele 194 (46.6%) was found to be flanked by 192 at a frequency of 22.6% [Sharma et al., 2003]. In the studied population from eastern India which is ethnically distinct from the North Indian population, the alleles 194 and 192 were present in almost equal frequencies of 43 and 41%, respectively. It is to be noted that the 192 allele, which is absent or occurs at a very low frequency in other populations constitutes a major allele in both the Indian studies and is probably associated with endogenous people of India. The 204 allele, which is present in Caucasian population but absent in Southeast Asian studies is detected in the North Indian study, and is present at a lower frequency in this study too, in agreement with the common origin of the Indo-European populations.

FRAXAC1 alleles distribution showed four different kinds of allele in our population with 154 being the most frequent like British (154) and North Indians (154) in contrast to the Thai (152), Chinese (152), Japanese (152). The relative abundance of the 152 allele shows a west to east gradient in India, having 16.5 and 31% frequency in the North Indian and present study, respectively. Heterozygosity of 51% was comparable to the British (50%) and Chinese (49%).

That populations in Southeast Asia and eastern India share some common genetic determinants is also evident from the distribution of beta globin mutations, and beta globin cluster haplotypes associated with the mutated hemoglobin HbE in these two populations [Bandyopadhyay et al., 1999]. Present data enforces the conclusion. The population studied here is an admixture of races where both Mongoloid and Dravidian ancestries are found. The population shows high degree of genetic complexity and ethnic diversity. The diversity is enhanced by human migration of African, West Asian, and European lineages into the northern, western, and eastern regions of the country which started from thirteenth century AD. The individuals included in our study are from Kolkata which is a metropolitan city where people from different caste and creed live. The genetic status of these individuals is thus extremely complex. This is exemplified in the distribution of beta globin gene mutations in this region, as we see that the HbE mutation prevalent in Southeast Asia

is present here at a high frequency, and mutations present in western part of the country are also present. Owing to these causes, it is not surprising that the allele frequencies observed in the three polymorphic loci DXS548, FRAXAC1, and FRAXAC2 show differences and similarities between the studied population and the major populations of the world, as has been discussed so far.

In the FRAXAC2 locus, six alleles were identified by us namely, 151, 152, 153, 154, 155, and 156. Studies on the English [Macpherson et al., 1994], Finns [Zhong et al., 1994], African-Americans [Crawford et al., 2000], population from Greece and Cyprus [Syrrou et al., 1996], and the northern population of India [Sharma et al., 2003] showed the most frequent allele to be 153 (4+), which is also the most frequent allele in our population represented in 42% of the normal population. The 154 (4) allele was found to be the most frequent allele in fragile X samples in this study and the North Indian study.

In the control group, the most significant haplotype was 194–154 (7C) represented in 44.4% of the X chromosomes followed by 8D (17.8%). In the fragile X group, the most frequent haplotype was 8C (22.7%) followed by 9D (18.2%). The 9D haplotype is absent in the control group and 8C haplotype is represented in only 6.7% of the X chromosomes. In fragile X group and in the normals, 9 and 10 different haplotypes were observed, respectively and there was no single major haplotype in each group as has been found in different ethnic groups of the world [Richards et al., 1992, 1994; Buyle et al., 1993; Macpherson et al., 1994; Zhong et al., 1994, 1999; Syrrou et al., 1996; Chiurazzi et al., 1996a,b] where founder effects have been demonstrated. Of the nine haplotypes represented in the fragile X group, four haplotypes were not present in the control group. This suggests the possibility of the admixture of immigrant haplotypes in our population, which is highly probable. Founder effect has been demonstrated for Asian population like the Chinese [Zhong et al., 1999] and Japanese [Richards et al., 1994] but it could not be demonstrated in the Thais [Limprasert et al., 2001] and in the ethnic population of Ashkenazi Jews [Pesso et al., 1997]. Despite the lack of single modal haplotype in this highly heterogeneous population, haplotype distribution between the controls and fragile X groups is significantly different ($P = 0.006$). This suggests weak founder effect, as argued by other authors [Chiurazzi et al., 1996c]. The haplotype distribution in chromosomes bearing >30 repeats is more like the controls ($P = 0.81$).

Given in Table III are the major haplotypes obtained in control and fragile X subjects in the major populations of the world and the studied population. We compared the common fragile X haplotype 8C with six other previous reports. The 6D haplotype has been found to be the most common fragile X founder chromosome haplotype in white Americans, Chinese, and British. But it has not been found among the fragile X chromosomes in ours and the North Indian study and in Thai population. The most frequent haplotype in Thai population is 7D, present in high frequency in both normal and fragile X chromosomes is absent in North Indian study. It occurs in this study at ~12% frequency.

Apart from the implication of allele distribution of DXS548, FRAXAC1, and FRAXAC2 loci on haplotype analysis, they can be correlated to the size of the CGG repeats. The highest number of repeats (≥30) has been identified in 12 normal individuals and was found to be associated with haplotypes 194–154 (30, 31, 32, 33, 34 repeats), 202–154 (31, 32), 198–154 (37), 192–154 (37), and 194–152 (37) show distribution pattern similar to the other normal alleles.

It is to be mentioned here that increase in heterozygosity in fragile X chromosomes is not obvious for founder effects. In the absence of founder effects when new mutations are frequent and haplotypes distribution of patients and normals would almost be identical and the observed high heterozygosity of fragile X chromosomes can be explained by their occurrence on different and distant haplotypes irrespective of their relative frequency in the normal population [Chiurazzi et al., 1996a].

In summary, the present work demonstrates a weak founder effect for fragile X full mutations and illustrates the variation of allele frequencies in different regions of India, when compared with other studies.

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TABLE III. DXS548–FRAXAC1 Haplotype Distribution in Major Populations of the World

Populations	Control (%)	Fragile X subjects (%)	Study reference
British[a]	194–154 (64.9)	196–152 (31.8)	Macpherson et al. [1994]
Finn[a]	196–154 (55.6)	194–154 (83.3)	Zhong et al. [1996]
Italy[a]	194–154 (63.9)	204–158 (24.0)	Chiurazzi et al. [1996a]
North Indians	194–154 (39.0)	194–154 (29.0)	Sharma et al. [2003]
East Indians	194–154 (44.4)	192–154 (23.8)	This study
Thai	194–152 (60)	194–152 (64)	Limprasert et al. [2001]
Chinese[a]	194–152 (54.9)	196–152 (62.5)	Zhong et al. [1999]

[a]Significant founder effect in population.

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