Asymptomatic carriers and gender differences in facioscapulohumeral muscular dystrophy (FSHD)


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Abstract

Facioscapulohumeral muscular dystrophy is an autosomal dominant muscle disorder, mapped to 4q35. It is characterized by remarkable inter- and intrafamilial clinical variability ranging from severe phenotype to asymptomatic carriers. The aim of the present study was to assess the size of the Eco RI fragment in a large sample of asymptomatic or minimally affected carriers as well as symptomatic patients, comparing both sexes, in order to verify if asymptomatic carriers are randomly distributed or concentrated in some particular families and if there is preferential parental transmission (maternal or paternal) resulting in non-penetrant carriers. We have analysed a total of 506 individuals from 106 unrelated families with at least one affected facioscapulohumeral muscular dystrophy proband. In all patients the molecular diagnosis was confirmed following double digestion (Eco RI/Bln I fragment <35 kb). About 20% among probands’ relatives who were found to carry the small fragment were asymptomatic or minimally affected, without preferential parental transmission, but with a significantly higher proportion of females (n = 37) than males (n = 14). Although asymptomatic carriers were found in about 30% of the families, some genealogies seem to concentrate more non-penetrant cases. A significant correlation between the size of the Eco RI fragment and severity of the phenotype was observed in the total sample but surprisingly this correlation is significant only among affected females. The gender difference in clinical manifestation as well as the observation that asymptomatic carriers are not rare should be taken into consideration in genetic counseling of affected patients or ‘at-risk’ relatives.

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1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant muscle disorder, mapped to 4q35 [1]. In most patients, probe p13E-11 (D4F104S1) detects a polymorphic Eco RI fragment smaller than 35 kb, which has 35–300 kb in normal individuals, and consists of multiple copies of a tandemly repeated 3.3 kb Kpn I unit. This unit has been termed D4Z4. The 4q35 region is highly homologous to the subtelomeric region of chromosome 10 (10q26) and molecular diagnosis is confirmed through the use of the restriction enzyme Bln I, which cleaves only the 10q26 units into small, non-detectable fragments [2]. The molecular mechanism that causes FSHD has been investigated for a long time. It had been suggested that deletions of integral number of the chromosome 4 units might affect nearby genes by altering the chromosomal structure, inducing position effect variegation. Recently, Gabellini et al. [3] reported an overexpression of genes upstream of D4Z4 in FSHD patients, which would be caused by the inappropriate transcriptional derepression of 4q35 genes inversely to deletion size. A polymorphic segment, distal to D4Z4, has also been found by Lemmers et al. [4], with two alleles: 4qA and 4qB. These authors observed that while in control individuals both alleles are equally present, only the 4qA allele is associated with FSHD. Clinically, FSHD is characterized by progressive weakness of facial, shoulder girdle and upper arm musculature. Lower limb involvement is not rare but there is a remarkable inter and intrafamilial variable expression ranging from asymptomatic carriers to severely affected cases.

Understanding the clinical variability in FSHD remains a great challenge. A correlation between the size of the Eco RI
fragment and clinical course has been observed in several studies with severe phenotypes being often associated with the smallest fragments [5–8]. On the other hand, the existence of abortive or partially affected cases as well as the gender difference in clinical manifestation has been recognized a long time ago [9,10]. However, we are not aware of any publication focusing mainly on asymptomatic or minimally affected carriers or gender differences in patients characterized at the molecular level. We have previously observed in Brazilian FSHD families a significantly greater proportion of females than males who remain asymptomatic or who are minimally affected [11]. Understanding the molecular mechanism, which protects some individuals who carry the FSHD fragment from clinical manifestation, is of utmost interest.

Therefore the aim of the present study was to: (a) assess the size of the Eco RI fragment in a large sample of asymptomatic or minimally affected carriers from FSHD families as well as symptomatic patients, comparing both sexes; (b) to verify if asymptomatic carriers are randomly distributed or concentrated in some particular families; (c) to verify if there is a preferential parental transmission (maternal or paternal) resulting in non-penetrant carriers.

In order to address these issues we have analysed and measured the size of the Eco RI fragment in a large sample of FSHD patients with a variable phenotype ranging from severely affected to asymptomatic.

2. Patients and methods

Patients from 106 unrelated families with at least one affected FSHD proband, referred to the Human Genome Research Center at the University of São Paulo, were analyzed. The probands and the at-risk relatives were clinically and neurologically examined by the same team, who have been working in our center for more than one decade. FSHD diagnosis was confirmed in all of them through molecular analysis. The clinical classification in the three clinical groups was done before the results of the molecular tests. The parents as well as other relatives of the probands were analysed whenever possible. All studies were performed following informed consent.

DNA was extracted from whole blood using standard techniques, digested with Eco RI and Eco RI/Bln I, separated in a 0.5% agarose gel for 72 h, transferred to a membrane by Southern Blotting and then hybridized with probe p13E-11 labeled with P32 dCTP [12].

In cases where the origin of the allele could not be confirmed or the fragment could not be sized through standard electrophoresis, pulsed field gel electrophoresis (PFGE) was performed in a 0.7% agarose gel, for 16 h, 6 V/cm, at 16°C. The blotting and hybridization procedures are the same as described earlier.

In all patients the molecular diagnosis was confirmed following double digestion (Eco RI/Bln I fragment <35 kb). For statistical analysis, patients (or at-risk relatives) were classified in three groups, according to the clinical presentation when last examined, as summarized below.

(1) asymptomatic or minimally affected (minimal weakness only in upper limbs with or without facial involvement). All individuals in this group were more than 30 years old. Individuals younger than 30 were considered pre-clinical and were not included in this study. This group was created after observation that some relatives, who were ascertained as clinically normal, had the deleted fragment.

(2) classical presentation: mildly affected with weakness only in upper limbs with or without facial involvement or moderately affected with muscle weakness in upper and lower limbs;

(3) severely affected: wheelchair confinement or severe childhood form with early onset (symptomatic before age 10).

Multiple comparisons were done through one-way analysis of variance (ANOVA) to test if different groups differ for the mean values of fragment sizes. When the variances of the fragment sizes differed significantly among the groups, the Welch’s correction for the F statistics (from the ANOVA) was used instead of the simple one-way ANOVA. The Tukey–Kramer honestly significant difference (HSD) test was employed to assess the contrasts, i.e. to verify if classes can be grouped in a way where the mean of each group is significantly different from the means of the others. This combination of tests was employed to verify if individuals from the three different classes of severity have different mean fragment sizes and also if there is a difference in the mean fragment size between genders from the same severity class.

Linear regression analyses were performed with the least squares method to verify whether individuals with shorter fragment sizes are more severely affected in the total sample and analyzing each gender separately. For this test, the severity classes were assumed to be a continuous variable. The Chi-square test was employed to compare the proportions of individuals among different groups. Calculations were performed with the JMP program (SAS Institute, 1994).

3. Results

A total of 506 individuals from 106 unrelated families with at least one affected FSHD proband were analysed. Molecular analysis revealed that 238 individuals carried a small Eco RI/Bln I fragment, with no gender differences (113 males and 125 females, $\chi^2 = 0.61; P > 0.05; 1$ d.f.). Among these 238 individuals 7 (1 male and 6 females) were mosaic and classified in group 1. As seen in Table 1,
the majority of the patients (n = 145; 61%) had the classical phenotype. Although in this group there were more males (n = 79) than females (n = 66) the difference was not statistically significant (χ² = 1.17; P > 0.05; 1 d.f.). A smaller proportion (17.6%) was severely affected, with a similar distribution for both sexes as well (20 males and 22 females; χ² = 0.09; P > 0.05; 1 d.f.). At the other end of the spectrum, 51 (21.4%) individuals were asymptomatic or minimally affected carriers. In this last group there were significantly more females than males (37 females: 14 males, χ² = 10.37; P < 0.005; 1 d.f.). This significant difference also holds if we use the empirical sex ratio in the total sample, instead of the 1:1 ratio (χ² = 11.06; P < 0.005; 2 d.f.). The asymptomatic group accounts for most of the global χ² (74%).

3.1. Phenotype severity versus fragment size

The assessment of the size of the Eco RI fragment including all patients in the three clinically classified groups confirmed that there is a significant correlation (F = 12.57, P < 0.001) between the fragment size and the severity of the phenotype (Fig. 1). Individuals with larger fragments tend to have the milder course while those who have the smaller ones are more severely affected. When genders are analysed separately, the correlation is significant for females (F = 9.26, P < 0.005) but no significant correlation between the disease severity and fragment sizes was observed for males (F = 3.01, P > 0.05).

The mean Eco RI fragment size was significantly larger in patients from group 1 than group 3 but there was a wide overlap in the distribution; no statistically significant differences were observed between groups 1 and 2 or groups 2 and 3 (Table 1).

A comparison between families with at least one asymptomatic carrier (n = 32) and families where all the analysed individuals carrying the FSHD Eco RI fragment (n = 74) were clinically affected showed that the mean Eco RI size was significantly larger in the first than in the second group (23 ± 5.9 kb and 20 ± 5.75 kb, respectively; P < 0.05).

On the other hand, the mean age at ascertainment including individuals carrying the small fragment showed that patients in group 2 (34.4 ± 12.3) were significantly older than those in group 3 (21.0 ± 18.5) (t = 5.52, P < 0.0001). In order to avoid a bias in the mean age estimates, the patients classified in group 1 were not included in the analysis because only individuals more than 30 years old were included in this group.

4. Discussion

4.1. Eco RI fragment size and clinical course

The significant correlation between the mean sizes of the Eco RI fragment and clinical severity in the total sample observed in the present study is in accordance with previous data [5–8,11]. However, surprisingly, when both sexes were analysed separately, the correlation was significant only for females.

It is noteworthy that a highly significant correlation between the deletion size and the severity of the phenotype was also observed only for females in spinal muscular atrophies (SMAs) [13].

![Fig. 1. Mean clinical severity and mean Eco RI fragment sizes. The correlation between the variables was significant in females (r = −0.251, P < 0.005) and in the total sample (r = −0.225, P < 0.0001), but not in the male sample (r = −0.162, P > 0.05). The lines correspond to the linear regression between fragment sizes and severity of individuals in the considered groups (total, males, and females samples). The dots correspond to the means of severity in the intervals of the horizontal axis and the bars represent the standard errors.](image-url)
The smallest fragment, of 10 kb, was observed in an isolated case, with the severe infantile form. Fragments of 12 kb were found in two brothers whose mother was a mosaic carrier and also in an asymptomatic female whose father had a classical course (at age 48 he was unable to raise up his arms and had facial and lower limbs weakness).

It is also noteworthy that the smallest fragments tend to be found among isolated or mosaic cases, rather than familial cases. On the other hand, if there were a correlation between the size of the Eco RI fragment and disease severity, an isolated case (or even familial cases) with a large fragment and a mild phenotype would have a greater chance to remain undetected. Indeed in the present sample 16 among 52 isolated cases (30.7%) have a severe phenotype while all cases classified in group 1 (asymptomatic or minimally affected) belong to families with at least one clinically affected relative.

On average the largest fragments were observed in the group of asymptomatic carriers but there were many exceptions since small fragments (15 and 18 kb, respectively) were detected in 5 (9.8%) carriers (3 females and 2 males) with no clinical signs. The possibility that these asymptomatic individuals are mosaic was excluded and since all of them have an affected relative with the same sized Eco RI/Bln I fragment, it is unlikely that this short fragment is on chromosome 10.

Ricci et al. [8] reported in their group of asymptomatic carriers a larger fragment size (ranging from 21 to 27 kb) but their study included only seven non-penetrant cases. In addition a great clinical variability was seen among patients carrying the same size fragment even within families, ranging from severely affected to asymptomatic. This observation is still more evident in families with a possible clinical anticipation [5,6] where the parental generation showed a much milder course than their affected offspring despite carrying apparently the same abnormal fragment.

4.2. Males versus females

Some authors suggested in the pre-molecular era that FSHD seemed to run a milder course in females than males [10,14,15]. This observation was supported in our previous study of 52 Brazilian families who were molecularly analysed and where we observed a significantly greater proportion of clinically affected males than females [11].

In the present study, the proportion of males (n = 113) versus females (n = 125) who inherited the abnormal fragment did not differ from the expected 1:1 ratio. In addition the mean size of the Eco RI fragment did not differ in males as compared to females in the total sample (21.37 in males and 22.29 in females, \( P > 0.05 \)) or in the three severity groups when analysed separately (\( P > 0.05 \)).

Among clinically affected patients (groups 2 and 3), even though there were more males (n = 99) than females (n = 88) the sex ratio did not differ statistically from expected. However, in group 1 (asymptomatic or minimally affected patients), there were significantly more females (n = 37) than males (n = 14) supporting our previous observation with a smaller sample [11]. Interestingly, in the pre-molecular era, Padberg [10] reported that among 107 studied patients the proportion of asymptomatic females (21/48 or 44%) was twice as high as for males (13/59 or 22%). Ricci et al. [8] also observed that 6 among 7 non-penetrant carriers in their series were females.

In addition, using pelvic girdle involvement as a criterion for the severity of the disease, Becker [15] observed that 80% of the males as opposed to only 23% of the females were severely affected. More recently, van der Maarel et al. [16] reported a gender difference among mosaic cases as well. In a survey of 35 de novo FSHD families, they observed that somatic mosaicism was present in 40% of cases, in either the patient or the asymptomatic parent. Interestingly while mosaic males were typically affected, mosaic females were asymptomatic.

4.3. Asymptomatic carriers

The proportion of relatives carrying the short fragment who are asymptomatic or minimally affected (51/238 or 21.4%) seems larger than the one reported in other studies [8,17]. One possible explanation is that we have analysed a greater proportion of probands’ first degree relatives who were apparently asymptomatic for two reasons: (a) Brazilian families are usually larger than European or North American families; (b) in the present series there is a greater proportion who were ascertained as apparently isolated cases than in other reports. Indeed in the publication of Upadhyaya et al. [18] only 27 among 130 families (~20%) were sporadic cases while in the series of Ricci et al. [8], among 122 families, 78 had multiple affected individuals while 44 (36%) were isolated cases. In the present study, 51 of 106 (48%) were apparently isolated cases although after molecular analysis we found 15 families with at least two individuals carrying the FSHD fragment. Therefore, only 36 of 106 (~34%) were indeed isolated cases, which is comparable with the observation of Ricci et al. [8] in the Italian population. It is noteworthy that Padberg [10] reported that 30% of FSHD cases he examined in the pre-molecular era were asymptomatic, a proportion even higher than the present one.

Another question that was also addressed is whether asymptomatic carriers are randomly distributed or concentrated in some families. In the present study we observed that the proportion of families with at least one asymptomatic carrier was 32/106 (30.18%).

However, it was observed that in some families there was apparently a greater proportion of asymptomatic or minimally affected patients. The distribution of patients according to clinical severity showed that 25 of the 51 asymptomatic cases were concentrated in only seven families, with fragment sizes ranging from 20 to 33 kb. Two particular very large families caught our attention.
In the first one, although the proband was a 29-year-old male with a classical presentation there were 10 individuals (4 men and 6 women) who were classified in group 1 and only four with a classical phenotype among 28 relatives who were tested. In this family the size of the fragment was 24 kb. In the second family, with a fragment size of 33 kb, even the proband was very mildly affected at age 47 although she had scoliosis since age 13. In this family 6 asymptomatic individuals were found to carry the small fragment (including the 72-year-old proband’s father) although 3 of them are younger than age 30 and could still manifest the disease later. Interestingly this family was ascertained because two sibs had another form of muscular dystrophy [19]. The observation of some genealogies with multiple asymptomatic carriers supports the hypothesis that epigenetic mechanisms (or modifier genes) protecting individuals from the deleterious effect of the FSHD allele would be more common in some particular families.

4.4. Parental transmission

It has been reported by us and others [5,20,21] that severe cases as well as the children of mosaic parents who are not sporadic cases are more often maternally inherited. This was confirmed in the present study since among 31 severely affected cases where it was possible to analyse the parental generation, 9 were due to new mutations, 17 (54.8%) were maternally inherited and only 5 (16.1%) of paternal origin. Interestingly, no statistically significant difference in the mean size of the fragment was observed in the maternally inherited cases as compared to the paternally inherited ones (23.7 ± 4.1 kb and 26.1 ± 4.7 kb, respectively, \( P > 0.22 \)).

On the other hand, among 22 cases classified in group 1 where it was possible to determine the origin of the FSHD fragment, 12 were maternally and 10 were paternally inherited. This observation suggests that, in contrast to severe cases there is no preferential transmission for asymptomatic or very mildly affected cases.

In summary, the main findings of the present study are:

(a) About 20% of individuals related to FSHD patients who carry a deleted Eco RI fragment remain asymptomatic or are minimally affected with a significantly higher proportion of females than males

(b) No preferential parental transmission was observed for asymptomatic carriers

(c) Asymptomatic carriers were found in about 30% of the families. However some genealogies seem to concentrate more non-penetrant cases, which supports the hypothesis that a ‘protecting mechanism’ (modifier gene or epigenetic) against the deleterious effect of the FSHD would be more prevalent in some particular families.

(d) A significant correlation between the size of the Eco RI fragment and severity of the phenotype was observed in the total sample. However, when both genders are analysed separately this correlation is significant only for females. This finding suggests that in addition to the hypothesis of Gabelini et al. [3] other genetic or epigenetic mechanisms would be modulating the severity of the phenotype.

(e) The gender difference in clinical manifestation as well as the observation that asymptomatic carriers are not rare should be taken into consideration in genetic counseling of affected patients or ‘at-risk’ relatives.

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