

# Improved molecular diagnosis of facioscapulohumeral muscular dystrophy (FSHD): validation of the differential double digestion for FSHD

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## Abstract

**A major advance in the molecular diagnosis of facioscapulohumeral muscular dystrophy is the recently reported elimination of confounding DNA fragments arising from homologous sequences located at 10q26. In order to evaluate the specificity and sensitivity of this important diagnostic test, we have compared a group of 130 patients fulfilling the diagnostic criteria for FSHD with 200 control subjects not known to have an increased risk of having an FSHD mutation. Among the FSHD cases the smallest *BlnI/EcoRI* fragment sizes ranged from 10 to >48 kb with 94.6% (95% CI 89.2-97.8%) of cases having fragment sizes of 34 kb or less. Among the 400 chromosomes from controls the smallest *BlnI/EcoRI* fragment observed with the *EcoRI/BlnI* double restriction enzyme digest was 38 kb  $\pm$  2 kb, suggesting a test specificity at a fragment size <34 kb of or very near to 100% (lower 95% CI 98.2%). Test sensitivity at <34 kb is estimated at 94.6% (95% CI 89.2-97.8%), all outliers having fragments >38 kb. The Southern blot analysis with DNA probe p13E-11 has created a valuable molecular diagnostic test for FSHD.**

(J Med Genet 1997;34:476-479)

Keywords: molecular diagnosis; FSHD; sensitivity

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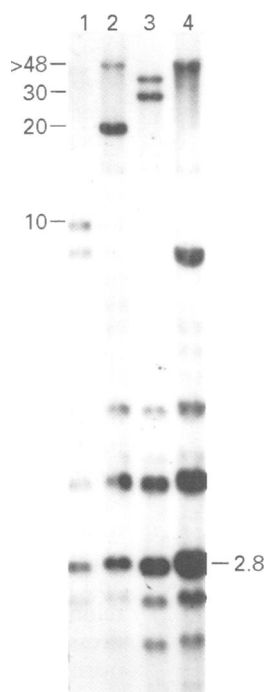
Received 27 August 1996  
Revised version accepted for publication 20 January 1997

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disorder characterised by progressive weakness and atrophy of the muscles of the face, upper arm, and shoulder girdle. The clinical features of the disease are variable, both within and between families. Disease onset is usually between the first and second decade, and gradually progresses with about 20% of patients eventually becoming wheelchair bound and with up to two-thirds of patients having disease related problems in their daily activities.<sup>1,2</sup> The gene for FSHD has been mapped to 4q35<sup>3</sup> and is closely linked to the locus D4F104S1 defined by the probe p13E-11.<sup>4,5</sup> In a small minority of FSHD families (estimated to be 5-10% at most), the disease locus may be unlinked to 4q35,<sup>6</sup> but no other specific chromosomal locations have yet been identified. To date, the molecular prediction of FSHD with D4F104S1 has been most

secure in those families which are linked to other 4q35 markers but has not permitted a molecular diagnostic test for general application.<sup>7</sup> Even in some 4q35 linked families with small D4F104S1 *EcoRI* fragments, testing has been complicated by recombinations occurring between the locus D4F104S1 and FSHD, with a maximum reported recombination fraction of 0.05.<sup>8</sup>

Using the restriction enzyme *EcoRI*, the probe p13E-11 identifies two highly polymorphic loci located at 4q35 and 10q26. The polymorphic *EcoRI* fragment at 4q is composed almost entirely of 3.3 kb *KpnI* tandem repeat units identified as D4Z4.<sup>9</sup> Among chromosomes from control subjects, the size of the 4q derived fragments usually ranges from 40 kb to approximately 200 kb,<sup>10</sup> whereas in two-thirds of FSHD patients, a deletion of an integral number of the D4Z4 repeats generates a shortened *EcoRI* fragment which is usually smaller than 28 kb.<sup>4,5</sup> Unfortunately, the size distribution of approximately 10% of *EcoRI* fragments from the homologous polymorphic locus at 10q overlaps with the range seen in FSHD cases,<sup>10</sup> and consequently many non-FSHD cases have *EcoRI* fragment sizes of less than 28 kb, thus giving a test specificity from *EcoRI* digest alone of 76% at this level.<sup>4,5</sup> It is evident that these smaller polymorphic *EcoRI* fragments arising from the 10q locus, coupled with the lesser risks of recombination and locus heterogeneity, create considerable difficulties when assessing the diagnostic significance of a shortened fragment in a person with clinical features suggestive of FSHD or in an at risk relative from a family with FSHD.

Recent detection of sequence divergence between the *KpnI* tandem repeat units located at 4q and 10q has shown a different distribution of restriction enzyme sites, and has shown that the enzyme *BlnI* specifically cleaves the 3.3 kb repeats derived from 10q, leaving intact the tandem repeat units at 4q.<sup>11</sup> Thus, a double restriction enzyme digest (DD) with the enzymes *EcoRI* and *BlnI* allows the specific detection of 4q fragments and should greatly facilitate the molecular diagnosis of FSHD. In order to define the sensitivity and specificity of this improved molecular test, we have re-screened samples from a group of previously diagnosed FSHD patients, as well as from a group of unaffected controls.



**Figure 1** The distribution of the smallest *EcoRI/BlnI* fragment sizes. Lane 4 contains DNA sample from a control subject. Lanes 1-3 contain samples from FSHD patients with smallest *EcoRI/BlnI* fragment sizes of 10, 20, and 30 kb respectively. The 9.4 kb band specific to the Y chromosome is observed in lanes 1, and 4. The presence of the 2.8 kb fragment is generated as a result of complete digestion of the 10q fragment with the enzyme *BlnI*.

### Subjects and methods

Affected cases were selected at random from familial and sporadic cases of FSHD studied over the past 10 years. The clinical record of each patient was reviewed and only those with an unequivocal diagnosis of FSHD or scapulo-humeral dystrophy were included.<sup>1,2,12</sup> One hundred and thirty unrelated FSHD patients were studied, of whom 27 represented new mutation cases with apparently normal parents. The control group comprised 200 subjects with no known background family history of FSHD. Blood was collected from all patients and controls and high molecular weight DNA isolated by use of standard methods. Using the same method previously used for the restriction enzyme *EcoRI*, 10 µg genomic DNA was simultaneously digested with *EcoRI* and *BlnI* (Amersham) for eight hours and then fractionated on a 0.5% agarose gel for 48 hours at 0.5 volts/cm. High molecular weight DNA markers (Gibco BRL) were used for accurate sizing of the double digested fragments (fig 1). The DNA on the gel was irradiated with 260 nm UV light for 60 seconds to induce random "nicks" throughout the DNA which was then transferred onto a Zetaprobe membrane (Bio-rad) by Southern blotting. The 800 bp insert from p13E-11 was labelled with [<sup>32</sup>P]dCTP using primer extension.<sup>13</sup> The posthybridisation wash was 2 × SSC, 0.1% SDS at 65°C as required, followed by autoradiography for one to seven days at -70°C using Fuji x ray film with an intensifying screen. To determine the accuracy of our assessment of fragment sizes, repeated measurements of the fragments of 30 affected subjects with shortened fragments and 105 controls with fragment sizes between 38

**Table 1** Details of subjects and families clinically affected with FSHD with an *EcoRI/BlnI* smallest fragment greater than 35 kb

FSHD family No	Smallest fragment size (kb)	Clinical details
221	38	Typical small FSHD family
260	38	Assumed sporadic case, onset in infancy
224	38	Typical FSHD, 2 affected members in 2 generations. Linkage of disease to 4q35 unclear although 38 kb fragment appears 4q35 linked
003	42	Brother and sister with typical but mild FSHD
016	42	"FSHD plus". Classic FSHD plus hypothyroidism or early cardiac death, separately, in daughters of historically affected mother
255	48	Sporadic FSHD with de novo 48 kb fragment
334	>48	Four affected sibs with typical FSHD, father very mild. Distant consanguinity (parents second cousins once removed)

and 48 kb showed a variation of reported fragment size that was ±1 kb for fragments up to 30 kb, ±2 for those from 30 to 40 kb, and ±3 for those greater than 40 kb. The autoradiographs were assessed blindly by two separate observers. A 95% CI (confidence interval) was calculated using the Clopper-Pearson exact method.<sup>14</sup>

### Results

Among those with previously diagnosed sporadic or familial FSHD, the size of the smaller fragment varied from 10 kb to >48 kb (fig 2). One hundred and twenty-three out of the 130 FSHD patients (94.6%) (95% CI 89.2-97.8%) had fragment sizes that were 34 kb or less. Among the seven cases with larger fragments, the fragment sizes varied from 38 kb (±2 kb) to >48 kb. The clinical details of each of these cases with larger fragments are summarised in table 1. Among 27 sporadic FSHD cases, the size distribution ranged from 10 to 48 kb (mean 17.8, SD 8.83), while the size distribution among 103 familial cases ranged from 10 to >48 (mean 22.0, SD 7.1) (p=0.01). Three fragment bands were evident in three unrelated subjects with FSHD. Among the control population, the smallest fragment size detected was 38 kb (±2 kb), while the majority had fragment sizes that were within the limiting mobility of the DNA on the agarose gel (>48 kb). Nineteen subjects had smaller fragment sizes that ranged between 38 and 48 kb (fig 2). In each FSHD patient with a double digest (*EcoRI/BlnI*) fragment of <28 kb, there was an approximately 3 kb reduction in the size of the double digest fragment compared to the *EcoRI* single digest fragment and without the appearance of other new fragments <48 kb.

### Discussion

Among the 400 control chromosomes, the smallest fragment observed with the *EcoRI/BlnI* double restriction enzyme digest was 38 kb (±2 kb), indicating that test specificity below this level is at or very near to 100% (lower 95% CI 98.2%). This is in contrast to the 76% test specificity associated with the presence of frag-

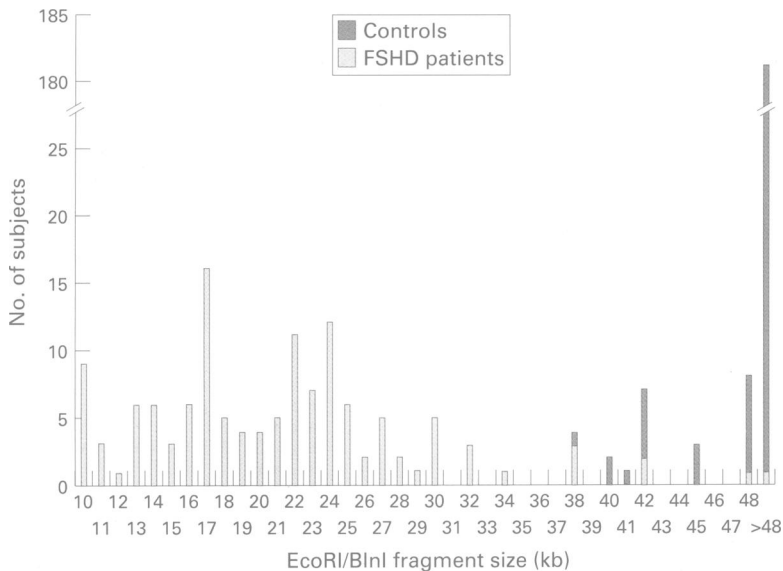


Figure 2 Size distribution of the smallest variable allele using *EcoRI* and *BlnI* restriction enzymes with D4F104S1 (p13E-11) in 200 controls and 130 unrelated FSHD patients.

ment sizes of 28 kb or less generated by a single *EcoRI* restriction digest.<sup>4,5</sup> Accordingly, these data confirm that *BlnI* cleavage of the 3.3 kb D4Z4 repeats derived from the 10q locus<sup>11</sup> greatly increases the test specificity associated with the detection of smaller 4q fragments. In addition, the data confirm the prediction of Deidda *et al*,<sup>11</sup> based on restriction site mapping, that among those affected with FSHD the size distribution of fragments created by *EcoRI/BlnI* double restriction enzyme digest is approximately 3-5 kb smaller than the previously observed size distribution associated with single *EcoRI* restriction digest.<sup>4</sup> Thus, there has been a useful additional separation of disease associated smaller fragments from the larger fragments found in normal controls, and at the 34 kb level, the test sensitivity is 94.6% (95% CI 89.2-97.8%), while specificity appears to be 100%. In addition, the test sensitivity and specificity estimates derived from these data remain unaltered in the range between 34 and 38 kb, indicating that the likely standard error associated with estimation of fragment sizes in this range will not have a significant impact on test accuracy, particularly when used with closely positioned standard high molecular weight DNA markers. While the 10q fragment has been successfully eliminated by the use of *BlnI* in this study, we would, however, still urge caution in this interpretation for diagnostic molecular testing, as a few exceptional alternatively defined apparent controls with *EcoRI/BlnI* fragments between 30-38 kb have been noted in other unpublished series from other centres (Naarden, personal communication, 1996). Of interest, in three unrelated FSHD patients, three alleles were observed following complete *EcoRI/BlnI* digestion. The occurrence of these additional bands among the FSHD affected group possibly suggests the presence of a previously unsuspected cross hybridising sequence or duplication of the 4q35 sequence, the significance of which remains unknown. While there was no evi-

dence of a third band among the control cases, such bands would only be apparent if their sizes were sufficiently small to be below the rate limiting mobility of the gel, or the possibility remains that the additional bands are only specific to FSHD. While this evidence, suggestive of the occurrence of a duplication, or of another cross hybridising sequence, does not influence the sensitivity and specificity of the test, it is being further investigated with pulsed field gel electrophoresis to determine the size and distribution of the larger fragments among those with three alleles.

The presence of a deletion of an integral number of D4Z4 repeats which do not appear to contain any expressed sequences<sup>9</sup> and are in close proximity to 4q telomeric sequences has led to the suggestion that the mechanism underlying the disease could be position effect variegation (PEV).<sup>15</sup> Experiments in *Drosophila* and yeast have shown that physical proximity to telomeric heterochromatin may actively suppress gene expression.<sup>16</sup> The occurrence of normal range fragment sizes in seven FSHD cases confirms that there is more than one mechanism responsible for FSHD. Disease phenocopies may occur with the presence of an indistinguishable muscular dystrophy possibly overlapping with another clinical phenotype. Diseases which may produce a very similar phenotype, although usually distinguishable by EMG and biopsy features, include spinal muscular atrophy and the scapulo-peroneal neuropathies.<sup>17,18</sup> Some cases, however, are not distinguishable even when the strictest of diagnostic criteria used in linkage studies are applied. Indeed, most linkage studies suggest that up to 5% of families are not linked to the 4q35 locus.<sup>6</sup> It is possible that some of these people may either have a mutation elsewhere in the genome (genetic heterogeneity) or have point mutations or other rearrangements in a separate 4q35 gene, located proximal to D4Z4.

This study has identified a potential subgroup of FSHD patients in whom there is no apparent correlation between smaller *EcoRI/BlnI* fragments and the expression of the disease. Reassessment of the seven unrelated FSHD patients with fragment sizes of 38 kb or more showed a range of unusual aspects, although all had typical features of FSHD that fell within the diagnostic criteria for FSHD. In one sporadic case with a fragment of 38 kb, onset was in early infancy and another subject had a de novo fragment of 48 kb compared to clearly distinguishable fragments >48 kb in the parents. In another family the mother was reported to have had typical but mild FSHD, one of two daughters with FSHD died at 17 years of age from cardiac failure, and in the other the FSHD was associated with treated hypothyroidism. Among the four familial cases, linkage data from at least one family suggest that it may not be linked to the 4q35 locus. Interestingly, the 38 kb fragment observed in this family is at the extreme low end of the range seen in the control population. In addition, six of the seven cases have fragment sizes that fall within the lowest 10% of the size range seen in the control population. Consan-

guinity was present in a family of four sibs each of whom had typical features of FSHD, in contrast to their father who was reported to be only mildly affected. While linkage data in this family were consistent with linkage to the 4q35 locus, the fragment size in this family was too large to be resolved (>48 kb). Explanation to account for these rare exceptions to the rule of deletion of D4Z4 repeats at 4q35 as the cause of FSHD must await eventual molecular definition of an expressed FSHD gene.

The difference between the mean small fragment size observed in sporadic and familial cases supports previous findings,<sup>19,20</sup> and also may prove useful for the diagnostic evaluation of apparently sporadic cases as they are more likely to have unambiguously small *EcoRI/BlnI* fragments.

In conclusion, these data clearly show that the Southern blot analysis with DNA probe p13E-11 using enzymes *EcoRI* and *BlnI* has greatly improved the molecular diagnostic test for FSHD. In FSHD patients with fragment sizes smaller than 35 kb, this test has a sensitivity approaching 95% while specificity approaches 100%. This represents a major step forward for both diagnostic confirmation as well as genetic counselling for those at risk of inheriting FSHD.

We are grateful to all referring clinicians for patient samples, in particular Drs A Fryer, A Colley, S Bundy, W Reardon, A Norman, T Cole, G Woods, A Hammans, J Harvey, N Haites, C Bennett, and A Green. We thank Dr R Newcombe for statistical assistance and the Wellcome Trust and AFM for their financial support.

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