Direct detection of 4q35 rearrangements implicated in facioscapulohumeral muscular dystrophy (FSHD)

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Abstract
The pl3E-11 probe has been shown to detect DNA rearrangements in sporadic and familial cases of FSHD. Its use, however, has been hampered by the fact that it detects at least two pairs of EcoRI alleles, one derived from the 4q35 region (D4F104S1), the other from 10q26 (D10F104S2). We have cloned pl3E-11 EcoRI fragments from the 4q35 and 10q26 subtelomeric regions and shown the presence of several restriction site differences within the KpnI tandem repeat units. The two loci present a different distribution of restriction sites for the enzyme BlnI which allows differential cleavage of the KpnI units derived from 10q26, leaving intact the 4q35 pair of alleles. This method of differential restriction greatly facilitates the interpretation of Southern blots obtained from affected and unaffected subjects, with an important improvement in reliability for diagnosis and genetic counselling. In addition, this method can be used to investigate the molecular mechanism of the 4q35 rearrangement implicated in the disease and to ascertain whether the rearrangement is because of interchromosomal exchange between 4qter and 10qter KpnI repeats. (J Med Genet 1996;33:361-365)

Key words: 4q35 rearrangements; facioscapulohumeral muscular dystrophy; BlnI restriction; DNA diagnosis.

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disorder which affects specific muscle groups and displays a variety of phenotypic expression. The gene for FSHD has been localised, by linkage studies, to chromosome 4q35 distal to the linkage group D4S171-F11-D4S163-D4S139.12 Wijmenga et al1 described a new probe, pl3E-11 (D4F104S1), telomeric to D4S139, which detects polymorphic DNA fragments in normal subjects ranging from 30 kb to 300 kb, de novo DNA rearrangements less than 28 kb in sporadic FSHD patients, and similar "small" fragments in familial cases. Physical mapping studies have localised the pl3E-11 probe on 4q35, close to a highly polymorphic tandem repeat unit defined by 3-3 KpnI restriction fragments.3 The most likely mechanism involved in the development of disease is a DNA rearrangement within the 4q35 locus resulting in deletion of a discrete number of tandem repeat KpnI units, with the appearance of smaller EcoRI fragments 10 to 28 kb in size.4 The probe shows two polymorphic loci and a 9-5 kb Y specific fragment. By haplotype analysis one of the loci could be assigned to chromosome 4q355 whereas the other locus segregates with 10qter microsatellite markers.6 Unfortunately 4q35 and 10qter p13E-11 alleles segregating in the same FSHD family sometimes overlap with each other on conventional agarose gel electrophoresis and make the interpretation of Southern blots difficult. In order to analyse the structural differences between the two pairs of p13E-11 alleles, we cloned the non-4q35 13 kb fragment segregating in a FSHD Italian family and a similar sized 4q35 fragment from a sporadic case of FSHD into a phage vector.7 Haplotype analysis with microsatellite 10qter markers and in situ hybridisation experiments with cloned KpnI repeats in different members of the Italian family have shown that the non-4q35 fragment is located in the 10qter region, in agreement with the haplotype assignment by the Leiden group.8 Restriction mapping of the 10qter clone, when compared with the 4q35 fragment, indicates a similar arrangement of KpnI tandem repeat units and flanking sequences. However, 4q35 and 10q26 EcoRI clones can be distinguished by restriction analysis with SfiI and StyI. We soon realised that this observation could be exploited for future applications in the field of molecular diagnosis and genetic counselling. We assumed that an accurate comparison of nucleotide sequences between 4q35 and non-4q35 EcoRI fragments would probably result in the identification of restriction enzymes able to cut specifically in either one of the alleles, facilitating the interpretation of the p13E-11 hybridisation patterns from the affected and unaffected members of FSHD families. Here we report that restriction enzyme BlnI cleaves the variable KpnI region of the 10qter p13E-11 fragments, allowing the direct identification of 4q35 alleles implicated in the disease.

Materials and methods
PATIENTS
4q35 haplotypes and p13E-11 fragments segregating in affected and unaffected members of family FSH21 are described in detail elsewhere.8 Family FSH46 is a sporadic family in which the affected son carries a de novo rearrangement of 13 kb, absent in both healthy parents.

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PULSED FIELD GEL ELECTROPHORESIS

High molecular weight DNA was isolated from freshly collected blood, or from EBV-transformed cells, and added to 0-5% Low Melt agarose (Biorad) according to the procedure described by Pharmacia LKB. Blocks were rinsed three times in sterile water and then equilibrated for 30 minutes at room temperature in the appropriate restriction enzyme buffer. Digestions with BhlI (Boehringer) and EcoRI (Biolabs) were carried out respectively in 200 μl with 60 U and in 400 μl with 150 U of enzyme. The enzymes were added in three equal portions at one hour intervals. After digestion, the blocks were rinsed three times in sterile water and then equilibrated for 30 minutes at room temperature in the electrophoresis buffer (0.5 × TBE).

Electrophoresis was performed at 12°C in a Pulsaphor electrophoresis unit with an HEX electrode (Pharmacia LKB); the agarose gel (Biorad) was 1-2% in 0.5 × TBE. The running procedure was carried out at 300 V in three steps: the first one was five hours with pulses of one second, the second three hours with pulses of 20 seconds, and the third two hours with pulses of 45 seconds. After electrophoresis, gels were stained with ethidium bromide and photographed; DNA was then transferred to Hybond N+ (Amersham) for hybridisation with α-32P ATP labelled p13E-11.

SOUTHERN BLOT ANALYSIS WITH 4Q35 MARKERS

Restriction of genomic DNA and hybridisation with LILAS (D4S163), pH30 (D4S139), and p13E-11 (D4F104S1) were as described in a previous paper. Digestion with enzyme BhlI (Boehringer) was carried out by incubating 10 μg of genomic DNA with 20 U of enzymes at 37°C overnight. Digested samples were separated by conventional agarose gel electrophoresis at 2 V/cm for 48 hours.

CLONING OF THE EcoRI p13E-11 FRAGMENTS INTO λ PHAGE

Genomic DNA (200 μg) was completely digested with EcoRI (Biolabs) according to the manufacturer’s instructions. The digested fragments were separated by size on a 10-40% sucrose gradient by centrifugation in a SW41 Ti rotor at 22,000 rpm for 22 hours. Fragments of the appropriate size were selected after agarose gel electrophoresis of single gradient fractions, pooled, and cloned into λ DashII/EcoRI (Stratagene). The ligation mixtures were packaged according to the Stratagene protocol and used to transform an E.coli ER strain (p2392). The bacteriophage plaques were transferred to nitrocellulose filters (Schleicher & Schuell) and hybridised with 32P labelled p13E-11. After rescreening DNA was prepared from the lysates of positive phases following PEG precipitation and purification through CsCl density centrifugation at 38,000 rpm for 24 hours using a SW50.1 Ti rotor. The positive clones isolated from the libraries constructed with the DNA of patients 21A and 46A are referred to as λ21A and λ46A.

RESULTS

SEQUENCE DIVERGENCE BETWEEN EcoRI 4Q35 AND 10Q26 CLONED FRAGMENTS IS OBSERVED WITHIN THE KpnI TANDEM REPEAT UNITS

We have already shown by restriction mapping of 4q35 and 10q26 cloned fragments from FSHD patients that restriction site differences exist between the KpnI tandem repeat units derived from different chromosomes: an additional site for SfiI has been described within the 10q26 KpnI units.7 A more accurate sequence comparison of the KpnI fragments showed that 10q26 alleles displayed new restriction sites for the enzymes SfiI and BhlI (C/CTAGG) that were absent in 4q35 homologues. In fig 1 a 300 nucleotide sequence derived from 4q35 D4Z4 units8 is compared with the corresponding tract derived from subcloned 10q26 KpnI units to show a 10qter specific BhlI site occurring about 80 nucleotides upstream of the KpnI site.

This observation prompted us to construct a detailed restriction map for BhlI using the phage clones derived from the two loci, with the aim of finding a restriction enzyme that could specifically cleave either one of the p13E-11 alleles on genomic DNA. SfiI cannot be used for this purpose since it cuts within the KpnI units of both alleles.

BhlI RESTRICTION MAPS OF PHAGE CLONED EcoRI FRAGMENTS DERIVED FROM 10Q26 AND 4Q35 REGIONS

Restriction maps were constructed after partial digestion with BhlI of cloned fragments. As reported before1 clone 46A contains a 13 kb
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EcoRI fragment derived from a 4q35 de novo rearrangement in a sporadic FSHD patient. Clone 21A contains a similar sized EcoRI fragment, unrelated to the disease, which segregates in patient 21A in association with 10qter microsatellite markers and has been mapped by in situ hybridisation to 10q26. As shown in fig 2, both 4q35 and 10q26 clones contain a common BlnI site about 3 kb upstream of the KpnI site in the first repeat unit. However, the 10q26 clone shows an additional BlnI site about 80 nucleotides upstream of the KpnI site of each repeat. The same results were obtained with BlnI restriction of the 43 kb cosmid clone, which contains at least 10 KpnI repeat units of the 10q26 type, indicating that the additional BlnI site is a constant feature of each 10q26 repeat unit. Therefore, we can assume that after double digestion of genomic DNA with the enzymes EcoRI and BlnI, the p13E-11 alleles derived from 4q35 will be shortened by 3 kb (the distance between the 5' EcoRI site and the first BlnI site), while the 10q26 alleles will be cleaved into BlnI fragments that will not show any hybridisation signal with p13E-11, except for a small piece of 2-8 kb spanning the distance between the first two BlnI sites.

**EcoRI**

**BlnI**

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**Figure 2** BlnI restriction maps of 4q35 and 10q26 phage clones derived from FSHD patients. Restriction sites: E, EcoRI; K, KpnI; Bl, BlnI. Each dotted bar represents the 3-3 kb repeat unit and the hatched box shows the position of probe p13E-11. Partial digests of i. clone DNA were hybridised with γ-ATPβγT7 and T3 oligomers.

**PULSED FIELD GEL ELECTROPHORESIS OF 4Q35 AND 10Q26 P13E-11 ALLELES AND EFFECT OF BlnI DIGESTION ON THE HYBRIDISATION PATTERN**

Fig 3 (panel A) shows a clear pattern of separation of the larger alleles by PFGE after EcoRI digestion of high molecular weight genomic DNA derived from lymphoblastoid cell lines of affected and unaffected members of family FSH21 (fig 4). PFGE separation allows the identification of at least three types of larger alleles, in the range of 140 kb, 85 kb, and 45 kb, but some bands appear to be doublets and one cannot assign these alleles with sufficient precision to either one of the chromosomes. Digestion with BlnI (fig 3, panel B) drastically changes the p13E-11 hybridisation pattern and indicates unequivocally the 4q35 origin of the multiple alleles, since the 10q26 fragments are cleaved. Fig 4 shows the pedigree of family FSH21 and the segregation of large and small p13E-11 alleles in association with 4q35 and 10q26 markers. It appears that the 4q35 rearrangement giving origin to the 23 kb fragment associated with the disease has occurred on the grandmaternal chromosome characterised by the 4q haplotype 85-11.5-8.2 (thin lined box) and is the result of a deletion of about 62 kb, corresponding to nearly 20 KpnI repeat units. The rearranged chromosome 4q35 has been transmitted to the affected daughter and her sibs, while the unaffected sister has inherited the chromosome characterised by the haplotype 140-8.8-7.2. At the same time the affected daughter has inherited the 10qter chromosome characterised by the haplotype 13-2-3 (thick lined box) carrying the small 13 kb fragment, while the unaffected sister has inherited from the mother the 10qter chromosome characterised by the haplotypes 85-1-3 carrying the large allele. In conclusion, BlnI digestion of genomic DNA converts the p13E-11 pattern from two loci (four alleles) to one locus (two alleles) and clearly identifies the...
Figure 4 Segregation of 4q35 and 10q26 markers in family FSH21. The two loci identified by p13E-11 are named D4F104S1 (chromosome 4) and D10F104S2 (chromosome 10). The thin lined boxes indicate the 4q35 haplotype associated with the disease, the thick lined box indicates the 10q26 haplotype segregating with the 13 kb fragment. The sizes of p13E-11 alleles were determined by pulse field gel electrophoresis as shown in fig 3. The 23 kb p13E-11 fragment originates from a 62 kb deletion occurring on the 85 kb grandmaternal allele.

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Figure 5 Effect of double digestion with EcoRI and BlnI on the sizes of p13E-11 alleles as determined by conventional agarose gel electrophoresis. Each panel refers to multiple alleles observed in subjects from different FSH families (see Results section for details).
unaffected mother (C) of the proband (A) and shows the same electrophoretic mobility of the rearranged 4q35 fragment transmitted from the affected father (B). The affected subject (A) appears to be homozygous for the fragment (see the double intensity of the signal) and this would prevent any prediction based on p13E-11 hybridisation pattern in the case of fetal DNA analysis. EcoRI-BlnI digestion cleaves the 23 kb allele derived from 10qter and only the 23 kb 4q35 fragments related to disease are preserved, allowing an unequivocal interpretation in the case of prenatal diagnosis. In conclusion, the conversion of p13E-11 hybridisation pattern to a single pair of 4q35 alleles in the 10qter region, a major diagnostic breakthrough was achieved by differential BlnI restriction of genomic DNA derived from affected subjects: the p13E-11 hybridisation pattern is changed from two loci (four alleles) to one locus (two alleles) with the removal of 10qter alleles interfering with the 4q35 disease related fragments. After double digestion with EcoRI and BlnI one can assume that the alleles migrating in the gel are exclusively 4q35 specific and the small allele undergoing a constant reduction in size (3 kb) is the rearranged fragment correlated with the disease.

Differential BlnI restriction can also be applied to investigate the molecular mechanism of the 4q35 rearrangements. Since we did not find, in any of the subjects examined, a size reduction larger than 3 kb, we can exclude that the DNA rearrangements are the result of cryptic translocations of 10qter sequences on the tip of chromosome 4. If this were the case, the size reduction would be increased proportionately to the number of 10qter KpnI units present in the hybrid rearrangement. It appears that the most likely mechanism is a 4q35 intracluster rearrangement involving homologous KpnI repeat units. As far as genetic counselling is concerned, the presence of a small 4q35 BlnI fragment will definitely prove that a deletion has occurred in a critical portion of the FSHD locus and the subjects carrying this fragment have a 95% risk of manifesting the disease in the first two decades of their life, taking into account the non-penetration of the gene in 5% of people. Linkage analysis with other 4q35 probes such as D4S163 and D4S139 will still be required to exclude the rare cases of FSHD unlinked to 4q35, to ascertain the presence and study the segregation of 4q35 haplotypes in the rare families where no small rearranged fragments are present.

It is worth noting that EcoRI-BlnI digestion will be particularly useful in genetic studies of small sized FSHD families where it is not possible to establish a significant linkage between small 4q35 fragments and the disease.

An extended application of BlnI restriction analysis in a larger number of FSHD families and normal subjects will confirm the association between short 4q35 fragments and the disease and the essential role of telomeric positional effect in the pathogenesis of the disease.

We wish to thank Dr I La Cesa and Professor C Scoppetta for clinical and diagnostic evaluation of the FSHD1 family members and Dr R Frants and Dr C Wiimenga for the gift of p13E-11 probe. This work was financially supported by grant 356 from Telethon-Italy and by Progetto Finalizzato Ingegneria Genetica, sottoprogetto 5.

Discussion

The use of p13E-11 for the diagnosis of FSHD found its specific application in the case of sporadic FSHD patients. The appearance of a small p13E-11 fragment in the proband, in the absence of any rearrangement in the unaffected parents, provided convincing evidence for a strict correlation between 4q35 rearrangements and the development of disease. In the familial cases, the results are not always reliable owing to the segregation within the same family of small fragments of different chromosomal origin (10qter mainly) in at least 10% of the world population. After cloning, restriction mapping, and partial sequencing of KpnI repeat units derived from the 10qter region, a major diagnostic breakthrough was achieved by differential BlnI restriction of genomic DNA derived from affected subjects: the p13E-11 hybridisation pattern is changed from two loci (four alleles) to one locus (two alleles) with the removal of 10qter alleles interfering with the 4q35 disease related fragments. After double digestion with EcoRI and BlnI one can assume that the alleles migrating in the gel are exclusively 4q35 specific and the small allele undergoing a constant reduction in size (3 kb) is the rearranged fragment correlated with the disease.

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