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# Monosomy of distal 4q does not cause facioscapulohumeral muscular dystrophy

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**Abstract**

**Facioscapulohumeral muscular dystrophy (FSHD) is a hereditary neuromuscular disorder transmitted in an autosomal dominant fashion. FSHD has been located by linkage analysis in the most distal part of chromosome 4q. The disease is associated with deletions within a 3·2 kb tandem repeat sequence, D4Z4.**

**We have studied a family in which an abnormal chromosome 4 segregates through three generations in phenotypically normal subjects. This chromosome is the derivative of a (4;D or G)(q35;p12) translocation.**

**Molecular analysis of the region 4q35 showed the absence of the segment ranging from the telomere to locus D4F104S1. Probe p13E-11 (D4F104S1), which detects polymorphic *Eco*RI fragments containing D4Z4, in Southern blot analysis showed only one allele in the carriers of the abnormal chromosome 4. Probe p13E-11 *Eco*RI fragments are contained in the subtelomeric region of 4q and their rearrangements associated with FSHD suggested that the gene responsible for the muscular dystrophy could be subject to a position effect variegation (PEV) because of its proximity to subtelomeric heterochromatin. The absence of the 4q telomeric region in our phenotypically normal cases indicates that haploinsufficiency of the region containing D4Z4 does not cause FSHD.**

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Facioscapulohumeral muscular dystrophy (FSHD) is a neuromuscular disease characterised by progressive weakness and atrophy of the facial and shoulder girdle muscles. The disease subsequently spreads to the abdominal, foot extensor, upper arm, and pelvic girdle muscles.[1 2] FSHD is transmitted by autosomal dominant inheritance. Its penetrance is almost complete and in 95% of the patients the onset of the disease is observed by the age of 20 with highly variable expression even within the same family, ranging from almost asymptomatic to wheelchair dependent patients.[3]

Its incidence, 1 in 20 000, is probably underestimated because of the nearly asymptomatic forms which could escape diagnosis. In the light of the number of de novo patients, the mutation frequency is high.[4 5]

The FSHD locus has been mapped by linkage analysis to the most distal part of 4q35.[6-12] Neither cytogenetic rearrangements linked to the disease nor deletions of the sole 4q35 band have been reported.[13] Larger deletions produce a distinctive malformation syndrome in which the phenotype correlates with the amount of chromosome material missing. Signs of muscular dystrophy have not been described in those patients.[14]

For this reason we have re-examined patients, collected in the Cell Repository of Biologia Generale e Genetica Medica, carrying balanced and unbalanced anomalies involving band 4q35.

Among them, we found a family in which an abnormal satellited chromosome 4, resulting from an unbalanced translocation with an acrocentric chromosome, segregates through three generations.

## Subjects, materials, and methods

### SUBJECTS

The proband is a 9 year old male, born at term, the first child of healthy, unrelated parents. Motor milestones were reached on time. At the age of 8 years he was referred to the Pediatric Clinic of the University of Pavia because of hypogenitalism. Cytogenetic analysis showed an abnormal satellited chromosome 4.

On the basis of the cytogenetic finding, a neurological examination was performed after informed consent was obtained. No muscular deficit was detectable in the upper limb girdle or in facial muscles. Osteotendineous reflexes were normal everywhere. He could easily climb stairs, jump, and walk both on his heels and on tiptoes, and stand up from lying.

Serum muscle enzyme levels were normal. Muscular sonography and electromyography performed on the femoral quadriceps, deltoid, and anterior tibialis were normal, as also were median, sural, and external sciaticus nerve conduction studies. CT scan of the brain was normal.

EEG showed diphasic spikes in the left centrotemporal regions and slow waves with spikes in the parietal, temporal, and anterior regions of both hemispheres. No seizures were reported.

The mother and the maternal grandfather, aged 30 and 61 years respectively, carry the same abnormal unbalanced chromosome 4. Neurological examination was entirely normal, with no sign of dystrophy. No miscarriages were reported in the family. The mother's sister has a normal karyotype.

*Summary of results*

| Locus | Distance* | Probe | Technique | Results | |
|---|---|---|---|---|---|
| | | | | Chr 4 | der (4) |
| ANT1 | | 168D11 | FISH | + | + |
| | nd | | | | |
| D4S187 | | Y28DD1 | FISH | + | + |
| | nd | | | | |
| D4S163 | | LILA5 | Southern | + | + |
| | 330 kb | | | | |
| D4S139 | | pH30 | Southern | + | + |
| | | M7 | FISH | + | + |
| | 250 kb | | | | |
| D4F104S1 | | I13G | FISH | + | − |
| | | p13E-11 | PFGE | + | − |
| | 211 kb | | | | |
| Telomere | | All human telomeres | FISH | + | − |

The loci are ordered from centromere to telomere as reported by Wijmenga *et al.*[16 17] *Data from Wijmenga *et al*,[16] Wright *et al*,[18] and Winokur *et al.*[19] nd indicates that the physical distance between the two adjacent loci has not been determined.

CYTOGENETIC STUDIES

Cytogenetic studies were performed on metaphase chromosomes obtained by standard methods from phytohaemagglutinin stimulated whole blood cultures and Epstein–Barr virus transformed lymphoblast cell lines.

Chromosome spreads were processed for QFQ, GTG, dystamicin-DAPI, and NOR bands. High resolution banding was obtained according to the technique of Dutrillaux and Viegas–Pequignot.[15]

IN SITU HYBRIDISATION

FISH experiments were carried out on mitotic preparations from the proband and his mother with the following probes: cosmids 168D11 (ANT1), M7 (D4S139), I13G (D4F104S1), and YAC Y28DD1 (D4S187) (table).

YAC 28DD1 was isolated by YAC Screening Centre DIBIT-HSR and IGBE-CNR (Milan, Italy) using D4S187 locus PCR primers (SBU 10 F-5′ ATTTGGTCCACCTTGTTCTCT 3′ and SBU 10 R-3′ CTTTGTCTCCCAAACATACATA 5′) and its chromosomal location was determined by in situ procedures.

Probes were labelled by nick translation with biotin-16-dUTP (Boehringer), purified through Sephadex G-50 in a Spin-X 1 ml column (COSTAR), alcohol precipitated with a 50-fold excess human placental DNA and a 50-fold excess salmon sperm DNA, and redissolved in 50% formamide, 20% dextran sulphate, 2 × SSC hybridisation mix at a final concentration of 50 ng/slide for cosmids and 200 ng/slide for YACs.

Hybridisation with All Human Telomeres probe (ONCOR) was carried out according to the supplier's instructions. The probes were denatured at 70°C for 10 minutes and then incubated at 37°C for 10 to 15 minutes to allow annealing of repetitive sequences. Chromosome slides were denatured at 70°C in 70% formamide, 2 × SSC for one minute, dehydrated with alcohol, and warmed at 37°C before hybridisation. Hybridisations were carried out for 16 hours in a moist chamber at 37°C for I13G, Y28DD1, and All Human Telomere probes and at 39°C for 168D11, M7 probes.

After hybridisation, different washing conditions were employed according to the probe used: for 168D11 and I13G 1 × 10 minutes in 50% formamide, 2 × SSC at 42°C and 1 × 10 minutes in 0·5 × SSC at 50°C; for M7 1 × 15 minutes in 50% formamide, 2 × SSC at 42°C and 1 × 10 minutes in 0·1 × SSC at 60°C; for Y28DD1 1 × 10 minutes in 50% formamide, 2 × SSC, 1 × 10 minutes in 1 × SSC, 1 × 10 minutes in 0·2 × SSC at 42°C; for All Human Telomere probe 3 × five minutes in 50% formamide, 2 × SSC at 37°C and 1 × five minutes in 2 × SSC at room temperature. Signal detection was achieved by treatment with three alternating layers of fluoresceinated avidin and biotinylated goat antiavidin (A-2011 and BA0300, respectively, Vector Laboratories). After the final avidin treatment, DAPI staining was carried out. The slides were finally counterstained with 0·5 μg/ml propidium iodide in phosphate buffered saline (PBS) for five minutes and, after a rinse in PBS, were mounted with an antifade solution (H100 Vector Laboratories).

PULSED FIELD GEL ELECTROPHORESIS ANALYSIS

EBV transformed lymphoblastoid cells were embedded in 1% LMP agarose gel (Boehringer) to a final concentration of $2 \times 10^7$ cells/ml. The agarose plugs were treated with proteinase K at a final concentration of 0·5 mg/ml in 50 mmol/l EDTA, 1% lauryl sarcosyl pH 8·0 overnight at 50°C with gentle shaking, extensively washed for 72 hours, and stored in 50 mmol/l EDTA at 4°C. Plugs were digested with the appropriate restriction enzyme and loaded on to a 1% agarose gel in 0·5 × TBE buffer. Samples were electrophoresed for 16 hours at 6 V/cm at 12°C. A LKB 2015 Pulsaphor apparatus (contour clamp configuration) was used under ramping conditions: two cycles of eight hours with pulses gradually increasing from one second to eight seconds. DNA was blotted on Nylon filter (Hybond N+, Amersham) according to the supplier's instructions.

SOUTHERN BLOT ANALYSIS

DNA from blood samples was extracted following standard procedures. Ten micrograms from each sample were digested with *Pst*I and *Taq*I restriction enzymes, fractionated on 1 × TBE 1% agarose gels for 18 hours at 50 V, and transferred onto Hybond N (Amersham) membranes by alkaline blotting.

RADIOACTIVE HYBRIDISATION

Probes p13E-11 (D4F104S1), LILA5 (D4S163), and pH30 (D4S139) were labelled by random priming with $^{32}$P dCTP, purified from unincorporated nucleotides by passing the reaction mixture through Sephadex G-50 in 1 ml spin column. An *Eco*RI blot was hybridised with probe p13E-11, *Pst*I blot with probe LILA5, and *Taq*I blot with probe pH30.

Hybridisation was carried out at 65°C in 0·5 mol/l $NaPO_4$, 7% SDS, and 1% BSA in a hybridisation oven.

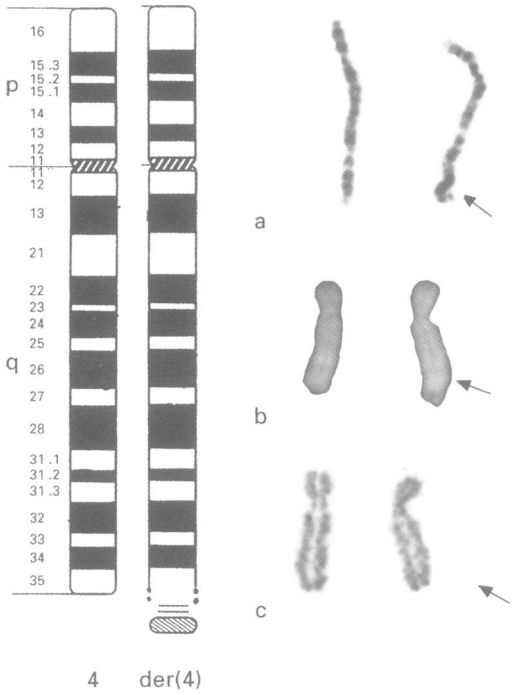


*Figure 1 Cutouts of the normal and the derivative chromosome 4 after high resolution banding (a), QFQ banding (b), and NOR staining (c). The arrows point to the satellites of the translocated acrocentric chromosome.*

Filters were washed at high stringency in 1 × SSC, 0·1% SDS at 60°C. Autoradiography was performed at −80°C for three days.

## Results

Analysis of GTG, QFQ, and NOR banded chromosomes from lymphocyte cultures of the proband showed the presence of an abnormal chromosome 4, derivative of an unbalanced translocation with an acrocentric. The origin of the satellites translocated onto chromosome 4 could not be determined since FISH with alphoid probes did not detect any centromeric sequences on the der (4q) terminal portion, and after distamycin-DAPI staining the satellites of this chromosome were negative, excluding their origin from chromosome 15.

The karyotype was interpreted: 46,XY, −4, +der (4), t(4;D or G) (q35;p12) (fig 1).

This abnormal chromosome was transmitted through the mother and the maternal grandfather. The proband's father and maternal grandmother were karyotypically normal.

The human telomeres probe, which hybridised to all telomeric regions, showed only one set of fluorescent spots at the telomeric region of the abnormal chromosome 4 long arm which corresponds to the telomere of the short arm of the acrocentric chromosome, confirming deletion of the telomere of 4q (fig 2a).

After FISH with cosmid I13G (D4F104S1) under the applied stringency conditions, fluorescent signals were present only on the normal chromosome 4 and not on the derivative chromosome 4 in 80 out of 115 metaphases analysed (fig 2b), in contrast with the hybridisation to both chromosomes 4 at lower stringency. No signals were observed on the short arms of the acrocentric chromosomes at the stringency condition applied. In 35 metaphases no hybridisation signals were detected.

In situ hybridisation with cosmids 168D11 (ANT 1), M7 (D4S139), and YAC 28DD11 (D4S187) showed positive signals on both chromosomes 4 (fig 2 e, c, d, respectively). Southern blot analysis with probes pH30 (D4S139) and LILA5 (D4S163) was infor-

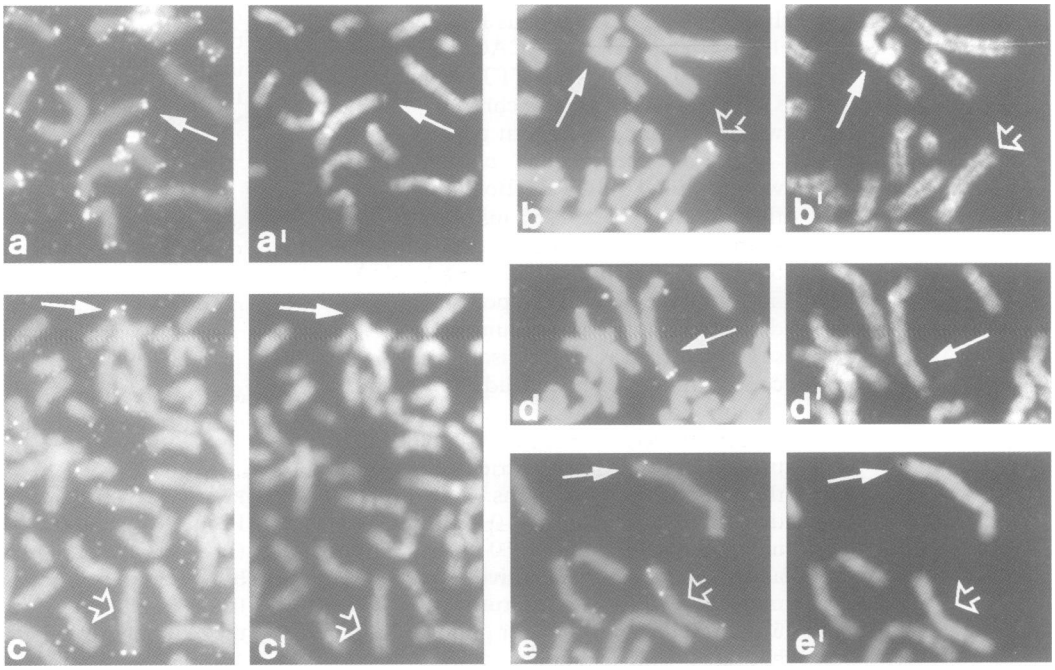


*Figure 2 FISH with All Human Telomeres probe (a) shows in the long arm of the derivative chromosome the presence of telomeric sequences only on the satellites of the translocated acrocentric indicating that the normal telomere of 4q has been deleted. FISH with cosmid I13G (b) fails to show positive signals on the abnormal chromosome 4 (full arrow). Hybridisation with cosmid M7 (c), YAC 28DD1 (d), and cosmid 168D11 (e) show positive signals on the normal chromosome 4 (open arrow) and the der(4) (full arrow). a', b', c', d', e': the same partial metaphases after DAPI staining.*

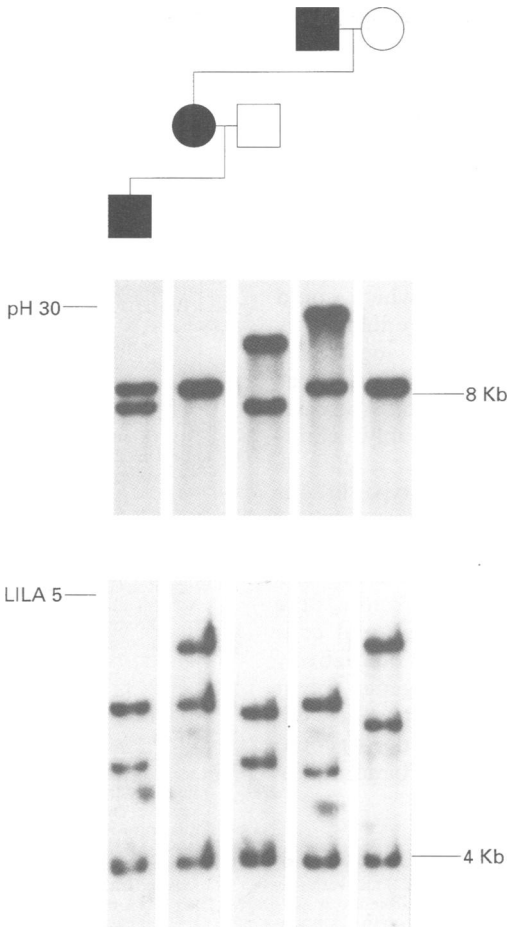


*Figure 3 Southern blot analysis of TaqI and PstI digested DNA with probes PH30 and LILA5, respectively shows the presence of alleles relative to loci D4S139 and D4S163 on the normal chromosome 4 and on the der(4). Carriers of the der(4) are indicated by closed symbols.*

mative and showed two alleles in the three subjects carrying the der(4) (fig 3).

Hybridisation with p13E-11 probe of the *Eco*RI PFGE blot showed the presence of only one chromosome 4 allele, which was of paternal origin, in the proband and a single, but different, allele in his mother (fig 4), confirming FISH data. The 23 kb allele present in the mother was inherited from the grandmother. The allele present in the grandfather was not present in the mother (data not shown).

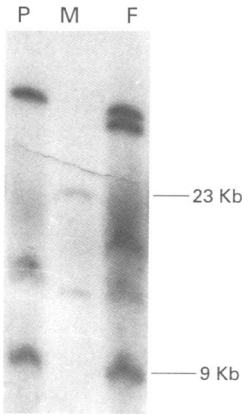


*Figure 4 PFGE blot analysis of EcoRI digested DNA of the proband (P), his mother (M), and his father (F) with probe p13E-11 (D4F104S1) shows the presence of only one allele in the proband and his mother. The 13 kb band is a constant band with PFGE analysis, and the 9 kb band is a constant male specific band.*

## Discussion

Results obtained from cytogenetic and molecular studies, summarised in the table, indicate that the der(4) present in the proband, his mother, and his maternal grandfather is deleted from the telomere up to the D4F104S1 locus, including the region containing the 3·2 kb tandem repeat as indicated by the absence of hybridisation spots using cosmid I13G in FISH analysis and the presence of only one allele on blot hybridisation with probe p13E-11.

This region, which is of particular interest in the search for the gene responsible for FSHD, shows high homology with the terminal long arm portion of chromosome 10, the chromosome 1 secondary constriction, and the heterochromatin of the acrocentric chromosomes.[19] This homology could be at the origin of the unbalanced translocation in our patients.

In 1992, Wijmenga *et al*[4] described probe p13E-11 which detects polymorphic *Eco*RI fragments of 10 to 50 kb in size. In familial and de novo FSHD patients the majority of *Eco*RI fragments segregating with the disease are smaller than in normal controls, ranging between 14 and 28 kb. However, among the British families studied by Upadhyaya *et al*,[20] 29% of FSHD cases showed fragments larger than 28 kb.

Cloning and analysis of p13E-11 *Eco*RI fragments obtained from FSHD patients and normal controls showed an internal segment consisting of 3·2 kb repetitive units detectable after *Kpn*I digestion, whose variable numbers could account for the polymorphism. The rearrangements associated with FSHD resulted from a deletion of integral copies of the 3·2 kb tandemly repeated units.[21]

The tandem repeat linked to FSHD (D4Z4) contains sequences homologous to repeats associated with heterochromatic regions of the human genome.[19 22] D4Z4 lies in close proximity to the 4q telomere[19 23] and, by direct visual hybridisation (DIRVISH), is adjacent to subtelomeric sequences.[23] On the basis of these observations, it has been postulated that a deletion of the tandemly repeated units could be responsible for *cis*-inactivation of the FSHD gene by a mechanism analogous to positional effect variegation (PEV).[19 22] PEV is a phenomenon described in yeast,[24] *Drosophila*,[25] and mouse,[26] in which gene expression could be altered by the structure of the heterochromatin situated nearby. PEV has never been found in humans, although its possible role in regulating gene expression has been postulated.[27 28]

The finding of three phenotypically normal subjects who are monosomic for the telomeric sequences and the heterochromatic subtelomeric region of chromosome 4 long arm indicates either that the gene is outside this region or a mechanism different from haploinsufficiency of the tandem repeat is the basis of the disease. Deletions in the repeat could result in an abnormal protein causing FSHD, while if the repeat is removed completely, as in our cases, there is no phenotypic consequence.

A similar situation is present in 4p16.3 region where deletion of distal 4p results in Wolf–Hirschhorn syndrome (WHS), characterised by severe mental and growth impairment, which does not show overlapping features with other autosomal dominant diseases mapped in the same region, such as Huntington's disease or chondrodysplasia.[29] In the first case the dominant inheritance is the result of haploinsufficiency of a gene(s) whose dosage is critical for normal development; in the other cases the disease is the expression of a mutant gene product. Analysis of the natural history of FSHD suggests that a mutated protein is more likely to be the basis of the disease; muscles are affected to a different degree and dystrophy is associated with sensorineural deafness and abnormalities of the retinal vessels,

showing that other tissues of different embryological origin can be involved in the disease. This peculiar phenotype could be the result of the expression of a mutant protein that can interfere with the normal metabolism of specific target cells. The response to the mutant protein could depend on the tissue cellular composition. FSHD shows variability in severity among patients and among different muscles. This could be explained either by the intrinsic differences of muscles which could react differently to the mutant product or by mitotic instability of the gene owing to the presence of tandemly repeated sequences, as in fragile X syndrome, Huntington's disease, myotonic dystrophy, and, probably, chondrodyplasia.

To explain the normal phenotype in our cases further hypotheses can be proposed. The deleted telomeric region on the der (4) has been replaced with another region containing heterochromatic sequences. Therefore, the FSHD gene may still be in a "normal condition" and not subjected to PEV. Alternatively D4Z4, a *cis*-regulating FSHD gene, might cause FSHD if altered, but not when deleted.

Cloning of the breakpoint of the der(4) could help in the search for the gene in band 4q35.

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