Identical de novo mutation at the D4F104S1 locus in monozygotic male twins affected by facioscapulohumeral muscular dystrophy (FSHD) with different clinical expression

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Abstract
Facioscapulohumeral muscular dystrophy (FSHD) is a progressive hereditary neuromuscular disorder, transmitted in an autosomal dominant fashion. Its clinical expression is highly variable, ranging from almost asymptomatic subjects to wheel chair dependent patients. The molecular defect has been linked to chromosome 4q35 markers and has been related to deletions of tandemly repeated sequences located in the subtelomeric region detected by probe p13E-11 (D4F104S1).

We describe a pair of monozygotic male twins affected by FSHD, carrying an identical de novo p13E-11 EcoRI fragment of paternal origin and showing great variability in the clinical expression of the disease, one being almost asymptomatic and the other severely affected. Their medical history was the same, with the exception of an anti-rabies vaccination performed at the age of 5 in the more severely affected twin. We hypothesise that the vaccination might have triggered an inflammatory immune reaction contributing to the more severe phenotype.

Keywords: FSHD; monozygotic twins

Facioscapulohumeral muscular dystrophy (FSHD) is a neuromuscular disorder characterised by progressive weakness and atrophy of the facial and shoulder girdle muscles, with subsequent involvement of the abdominal, foot extensor, upper arm, and pelvic girdle muscles.1,2 The incidence of the disease, which is transmitted in an autosomal dominant fashion, is estimated as 1 in 20 000. Its penetrance is considered almost complete and in 95% of patients onset is by the age of 20 years. Its clinical expression is highly variable even within the same family, ranging from nearly asymptomatic to wheelchair dependent patients.3

Pathological alterations in muscle biopsies are consistent with a primary defect of muscle

Figure 1 Three generation pedigree of the family. The haplotypes obtained with four different 4q35 marker loci (D4S171, D4S163, D4S139, D4F104S1) are indicated below the symbols. A recombination event between loci D4S139 and D4F104S1 has occurred during maternal meiosis in II.3. II.3 shows the paternal haplotype carrying the de novo mutated allele at the D4F104S1 locus and indicates that the mutational event has occurred during gamogenesis in the grandfather or postzoogetically, before the twinning, being associated with alleles 153, 1, and 4 at D4S171, D4S163, and D4S139, respectively. Locus D4F104S1 allele size range: a, larger than 48 kb; b, between 38 and 48 kb; c-d, between 33 and 38 kb; e, smaller than 23 kb. Closed symbols indicate the affected monozygotic twins (II.5 and II.6).
Monozygotic twins with FSHD

Figure 2. Southern blot analysis of EcoRI digested DNA with probe p13E-11 shows the presence of a de novo allele, shorter than 23 kb, in both twins (II.4 and II.5) and in one son of II.4 (III.2). Subjects affected by FSHD are indicated by closed symbols.

fibres, including variation of fibre diameter, centrally placed nuclei, occasional necrosis of muscle fibres, and increased endomysial and perimysial connective tissue. Occasionally, mononuclear cells may be detected and inflammatory changes have been described in 40-80% of FSHD patients.

The FSHD locus has been mapped by genetic linkage analysis to the chromosome 4q35-qter region. Specific deletions of chromosome 4q35 have been detected using probe p13E-11 (D4F104S). These deletions occur in highly polymorphic EcoRI fragments displaying a VNTR-like structure with a 3.3 kb tandem repeat unit. It has been postulated that deletions of the tandemly repeated unit, which have been localised to the subtelomeric heterochromatin, may be responsible for the cis inactivation of the FSHD gene by a mechanism similar to the positional effect variegation. Alternatively this structural modification could influence the regulation of gene transcription. However, no candidate gene has yet been identified in the FSHD region. Recently, probe p13E-11 has shown that fragments with a similar 3.3 kb repeat polymorphism occur on chromosome 10qter and that 10% of the 10q linked polymorphic fragments are smaller than 35 kb, a size comparable to the 4q deleted fragments observed in FSHD patients. The 10q linked fragments are distinguishable from the 4q linked fragments by double digestion of the DNA with EcoRI and BlnI.

To date three sets of monozygotic twins affected by FSHD have been described; one pair was discordant for the disease and only the affected twin showed a deleted EcoRI fragment at the D4F104S1 locus. In the two other sets FSHD was familial and dominantly inherited in the concordant twins. Here we describe two monozygotic male twins affected by FSHD, carrying an identical p13E-11 EcoRI fragment of “de novo” origin and showing a great difference in the clinical expression of the disease.

Materials and methods

Patients

The twins, CE and CB, were born at term on 19 July 1952 after an uneventful pregnancy and a normal delivery. They were raised together and their psychomotor development was normal. The twin II.6 (fig 1) at the age of 5 years underwent a rabies vaccination, at that time (1957) performed with a nerve tissue vaccine (NTV). He had complained of weakness at the age of 12 years, followed by progressive difficulty in his motor performance, hypotrophy of the biceps, and upward slanting of the scapulae. At the age of 32, muscle weakness extended to the lower limbs, mostly to the gluteus muscles. He showed a marked lordotic posture and had difficulty in getting up from the floor. Neurological examination at the age of 40 showed hypotonia and wasting in the upper limbs and quadriceps and a marked lordosis. His gait was unstable with a tendency to fall frequently. He was unable to walk on tiptoe. The facial muscles were involved with inability to blow out the cheeks and whistle. Distal power was conserved, although mildly reduced in his hands. He has two sons (III.4 and III.5, fig 1), aged 15 and 9 years, who are completely asymptomatic with a normal neurological examination.

Twin II.5 (fig 1) was almost asymptomatic. At the age of 15, neurological examination showed a mild hyperlordotic posture, upward slanting of the scapulae, and mild hypotrophy of the biceps. At the most recent examination
in 1996, clinical progression had not occurred. No sign of muscular dystrophy was present in the lower limbs. He was still able to whistle but showed a mild inability to screw his eyes up tightly. He has two children (III.2 and III.3, fig 1), aged 14 and 11 years, who are completely asymptomatic, with the exception of mild winging of the scapulae in both.

The family history was negative for the presence of neuromuscular diseases on the basis of clinical history. The twins' father (I.1) was asymptomatic. His neurological examination showed mild winging of the scapulae without weakness. His father has been reported to show the same posture and gait. No clinical records are available for him.

**MOLECULAR TECHNIQUES**

DNA extracted from peripheral leucocytes of all subjects was studied for allele segregation at loci D4S163 (probe LILAA/PsiI), D4S139 (probe pF300/TaqI), and D4F104S1 (p13E-11/EcoRI) using standard electrophoresis and Southern blot techniques. The size of the fragments at D4F104S1 was allocated by comparison with high molecular weight DNA standards (Gibco BRL) of size 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb on the same gel. The de novo p13E-11 EcoRI fragments were assigned to chromosome 4 by double digestion with EcoRI/BlnI.

(CA)n dinucleotide Mfd22 (DAS171 locus) was tested using published primer sequences.22 PCR amplification was carried out in a total volume of 15 μl containing 100 ng of genomic DNA in 50 mmol/l KCl, 50 mmol/l Tris-HCl (pH 9.0), 7 mmol/l MgCl₂, 5 pmol of each primer, 0.2 mmol/l dNTPs, 0.2 mg/ml BSA, 16 mmol/l (NH₄)₂SO₄, and 1 U of Taq polymerase (SuperTaq, HT Biotechnology Ltd, Cambridge, UK). PCR conditions consisted of 27 cycles of one minute at 95°C, two minutes at 55°C, and one minute at 72°C for each cycle.

The PCR products were run on a denaturing 6% polyacrylamide gel in 0.5 × TBE. Monomoyosy was proven in the twins by using DNA typing. Forty loci were analysed with probes 33.6 and 33.15.21,22 Hybridisation was carried out on HindIII digested DNA from both twins in a 0.5 mol/l NaPO₄, 7% SDS, 1% albumin, and hybridisation mixture at 65°C. Washes were performed at 60°C in 0.1 × SSC, 0.1% SDS.

Muscle biopsies were taken from the deltoid muscle and frozen in liquid nitrogen. Cryostat sections were stained with standard histological techniques and immunocytochemically with antibodies to a variety of proteins. These included dystrophin, spectrin, sialoglycans, utrophin, fast, slow, and fetal isoforms of myosin, laminin, α1, α2, β1, and γ1 chains, and HLA class I antigens. Primary antibodies were incubated for 30 minutes at room temperature and visualised with a biotin-streptavidin-Texas red method, as previously described.

**Results**

Molecular analysis of the D4S171, D4S163, D4S139, and D4F104S1 loci was extended over three generations in the family, including the parents and the children of the twins (fig 1). Probe p13E-11 showed a de novo fragment of about 23 kb at the D4F104S1 locus in both twins, which was transmitted to one child (III.2, fig 2). Haplotype segregation studies (fig 1) indicated that the de novo fragment originated from a mutational event occurring during paternal gametogenesis, since in III.3 the grandparental haplotype is associated with the de novo fragment. Haplotype analysis also showed that in subject II.2, a recombination event between D4S139 and D4F104S1 had occurred during maternal gametogenesis. We were able to confirm the origin of the de novo p13E-11 EcoRI fragments at the chromosome 4 D4F104S1 locus by double digestion with EcoRI/BlnI (fig 3). As reported by Deidda et al.,10 the 10q linked fragments are distinguishable from the 4q linked fragments by double digestion of the DNA with EcoRI and BlnI. After this double digestion p13E-11 4q linked fragments are smaller than the EcoRI fragments, whereas the 10q linked fragments are not detectable after electrophoresis at the standard conditions, owing to a high number of BlnI restriction sites in the 10q repeat units.

Using the two minisatellite probes (33.15 and 33.6) monomoyosy (fig 4) was confirmed, compared to a probability of chance association of <5 × 10⁻⁶. Paternity was confirmed with traditional and molecular markers (probability of paternity W=99.999% according to Essen-Moller).26

The muscle biopsy of the asymptomatic twin showed no significant features. There was no abnormality in fibre size, no increase in connective tissue, and no structural changes. Immunocytochemistry also showed no abnormalities and the expression of all proteins studied was normal. Fetal myosin, usually associated with regenerating fibres and thus an indicator of previous muscle damage, was not detected in any fibres. Expression of HLA class I antigens was also normal and confined to the vascular components (fig 5A, C, E).

The biopsy of CE, in contrast, showed variation in fibre size with several very small fibres. Occasional necrotic fibres, a little connective tissue, and a few infiltrating cells were also seen. Immunocytochemical labelling for spectrin, dystrophin, the dystrophin associated glycoproteins, and laminin chains was normal. Slight traces of utrophin and HLA class I antigens were detected in some mature fibres. Several of the small fibres also showed HLA expression but these also expressed fetal myosin and were probably immature. Fetal myosin expression was mainly confined to the small fibres (fig 5B, D, F).

**Discussion**

We have studied a pair of monozygotic twins referred to us for clinical and molecular assessment of PSHD. They are genetically identical and both show a de novo p13E-11 EcoRI fragment associated with chromosome 4. Neurological examination of their parents was normal, with no sign of muscular dystrophy.
presence of a de novo short p13E-11 EcoRI fragment only in the affected twin, presumably owing to a postzygotic mutation and thus explaining the phenotypic differences between the two brothers. The two other pairs were monozygotic female twins with different clinical expression and carrying a similar EcoRI fragment at the D4F104S1 locus. Other chromosome 4q35 specific markers were not tested. No specific anamnestic records were reported. On account of the gender of the patients, the authors considered that X inactivation might potentially influence the expression of the disease in these subjects. This hypothesis does not apply to our cases as the monozygotic twins described here are male. They carry the same mutated fragment but show a different phenotype.

Clinically discordant monozygotic twins for various diseases have often been reported. Discordance is particularly evident, for example, in Wiedemann-Beckwith syndrome (WBS); out of the eight cases of monozygotic twins described,27–29 seven were discordant for the expression of this syndrome, showing one twin with many classical manifestations of the disease and the other having few or none. Minor anomalies observed in most of the normal co-twins were ascribed to the presence of mild forms of WBS and have been explained as a consequence of either environmental factors or somatic mosaicism. In our case a postzygotic mutation, occurring before the twinning and leading to a different extent of mosaicism in the two brothers, might account for the differences in the clinical manifestation of the muscular dystrophy. However, qualitative evidence of mosaicism, as shown by the presence of an additional less intense band, was not detected by probe p13E-11.

Considerable phenotypic variability is described among FSHD patients with wide variation in the age of onset and in the rate of progression as well as in the extent of specific muscle involvement. In an attempt to explain these clinical differences, Lunt et al.28 studied a large group of familial cases and found a correlation between the p13E-11 EcoRI fragment size and age at onset or progression of muscle impairment. The smallest fragments were associated with the earliest age of onset and with earlier use of a wheelchair. The authors proposed that D4F104S1 fragment size determines a part of the variance in age of onset and eventual severity of FSHD. Furthermore, the analysis of the age at onset of clinical signs in patients from multigenerational families suggested that anticipation could occur in FSHD. In standardised genotype-phenotype studies it has been shown that the degree of clinical severity present in the parent is significantly lower when compared to the offspring, supporting the hypothesis of anticipation in FSHD. Nevertheless, we have studied families in which almost asymptomatic subjects and wheelchair dependent patients were present within the same generation, with affected members carrying the identical EcoRI fragment (unpublished observations). In this respect, FSHD also differs from those hereditary

Haplotype analysis of the family showed that the de novo rearrangement in the D4F104S1 locus had occurred in paternal gametogenesis or postzygotically in the paternal chromosome 4 before the twinning and that the mutated fragment was subsequently transmitted to one of the grandchildren (III.3, fig 1). The genetic identity of the two twins does not match their clinical picture, with twin CB being almost asymptomatic and CE severely impaired.

To our knowledge, three sets of monozygotic twins affected by FSHD to a different extent have been reported. In one pair, molecular analysis of the D4F104S1 locus showed the

Figure 5. Immunolabelling of the biopsies from CB (A, C, E) and CE (B, D, F) with antibodies to C-terminal dystrophin (A, B), HLA class I antigens (C, D), and fetal myosin (E, F). Note the greater pathology in CE and the very small fibres that express fetal myosin and HLA class I antigens, suggesting that they are regenerating fibres. Note also the mild expression of HLA class I on mature fibres in CE but its absence from fibres in CB (the fine peripheral dots in E and F are the result of autofluorescence of lipofuscin). Bar≈50 μm.
diseases characterised by meiotic and mitotic instability resulting in an expansion of triplets interfering with gene expression. In FSHD patients, deletions of tandem repeats occur in the heterochromatic subtelomeric region of chromosome 4q. Structural changes of the heterochromatin could interfere with the expression of genes located proximally to this region. However, this hypothesis does not explain the clinical variability of the disease within the same family, being the deleted fragments transmitted without any modification of their length. To clarify the basis of the great variance in the levels of expression of the disease, a more complex mechanism should be considered. In this respect, twins studies could be very useful in order to separate the aspects linked to the genetic background, and therefore to the natural history of the disease, from those related to environmental factors that might have influenced the phenotype.

There was no difference in the clinical history of our patients, with the exception of an anti-rabies vaccination performed with a nerve tissue vaccine (NTV) in CE at the age of 5 years. Furthermore, their father and paternal grandfather had sloping shoulders, causing an FSHD-like posture, suggestive of the presence of an almost asymptomatic form of the disease in the previous generations. If this were the case, linkage analysis would confirm the segregation of FSHD with the putative 4q35 locus, while the de novo ezrin fragment of paternal origin present in the twins would represent a mutational event independent of the transmission of the disease and not interfering with its clinical severity.

Inflammatory pathological findings are common in several primitive muscular dystrophies. In view of a possible role of inflammation in the clinical picture of FSHD, Arahata et al. analysed inflammatory mononuclear cells in FSHD muscle sections and in normal controls. They found that an increased number of necrotic fibres corresponds with an accumulation of inflammatory cells, and hypothesised that mononuclear cellular infiltrates may enhance muscle fibre damage. The muscle pathology in case CE shows features consistent with FSHD and no specific defects in protein expression were seen. It is interesting to note, however, that there was some expression of HLA class antigens in the muscle fibres. This is a common feature of inflammatory autoimmune myopathies. In our case, the anti-rabies vaccination administered to CE could have triggered an immune reaction that may have contributed to the more severe phenotype.

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