A large patient study confirming that facioscapulohumeral muscular dystrophy (FSHD) disease expression is almost exclusively associated with an FSHD locus located on a 4qA-defined 4qter subtelomere.

Thomas NST¹, Wiseman K¹, Spurlock G¹, MacDonald M¹, Üstek D² and Upadhyaya M¹

¹ Department of Medical Genetics, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK.

² Division of Medical Genetics, Department of Pediatrics, Istanbul University, 34390, Capa, Istanbul, Turkey.

Correspondence to: Professor Meena Upadhyaya, Department of Medical Genetics, School of Medicine, Cardiff University, Heath Park, CF14 4XN, UK; Email: upadhyaya@cardiff.ac.uk
Abstract

Introduction: Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant muscle disorder that represents the third most common human muscular dystrophy. The FSHD disease locus is located at chromosome 4q35, where it is associated with large contractions of locus D4Z4, a highly polymorphic repeat array. In addition to the almost complete association between FSHD disease expression and the presence of large D4Z4 deletions, another biased molecular association with a specific 4qter subtelomeric sequence has recently been described in FSHD patients. Two distinct 4qter subtelomeres, defined as types 4qA and 4qB, have been identified, and these two allelic forms were found to be equally prevalent in the Caucasian population. However, in almost all chromosome 4-linked FSHD patients, disease expression is only found to occur when large, 4q35-located D4Z4 deletions are situated, in cis, on 4qA-defined 4qter subtelomeres.

Objective: To confirm and extend this FSHD disease association data by measuring the frequency of type 4qA- and 4qB-defined 4qter subtelomeres in a large cohort of 164 unrelated FSHD patients, all with known large D4Z4 deletions, derived from Turkey and the UK.

Results: An almost complete association was found between large, 4q35-located D4Z4 deletions located on 4qA-defined 4qter subtelomeres and FSHD disease expression in our extensive patient cohort. In addition, the failure of probes 4qA and 4qB to hybridise to two patient-derived DNA samples, identified the presence of an additional rare type of 4qter subtelomeric sequence in humans.

Conclusions: We have greatly strengthened the FSHD disease association data, using the largest FSHD patient panel so far studied, to confirm that large D4Z4 deletions located in cis on 4qA-defined 4qter subtelomeres are essentially in complete association with FSHD disease expression. This confirmed data will have immediate practical value for the molecular diagnosis of FSHD in suspected patients.

KEY WORDS: 4qA-defined 4qter subtelomere; D4Z4 repeat array; FSHD disease expression; Facioscapulohumeral muscular dystrophy; Genetic diagnosis
INTRODUCTION:

Facioscapulohumeral muscular dystrophy (FSHD), an autosomal dominant muscle disorder in which patients display a progressive weakness of the musculature of the face, shoulders, and upper arms, [1] it is the third most common human muscular dystrophy. FSHD has an estimated incidence of at least 1:20,000 livebirths and exhibits a high level of new mutations, which represent 10-30% of all new FSHD cases.[2] A high degree of intra- and inter-familial clinical variability is exhibited by patients, with affected individuals often expressing a range of clinical phenotypes, from severe neonatal-onset forms, through ‘classical’ FSHD, to asymptomatic or non-penetrant gene carriers, sometimes all within the same family.[3]

The FSHD locus is located at 4q35 adjacent to the 4qter subtelomeric region.[4] A variable number tandem repeat polymorphic locus, designated D4Z4, was identified in this region, which in normal individuals usually contains multiple integral copies (11 > 100) of a 3.3kb KpnI tandem repeat sequence.[5] In the majority of FSHD patients however, large integral deletions of individual KpnI repeats reduce the size of this D4Z4 repeat arrays down to less than 11 repeats (<38kb). No specific FSHD gene has yet been identified, nor has the underlying pathological mechanism(s) involved in disease expression been explained. An approximate inverse correlation has been reported [6][7] between the exact number of D4Z4 repeats retained by a patient and the level of disease severity exhibited by that patient, although the clinical variability associated with FSHD may often confound this relationship.

Two distinct 4qter subtelomeres, designated 4qA and 4qB, have been recognised [8][9] and are identified by hybridisation of specific 4qA and 4qB probes to genomic sequences located immediately distal of the D4Z4 repeat array. The 4qA-defined 4qter subtelomere contains an ~8.0kb β-satellite sequence (Fig. 1) that demonstrates significant sequence homology to the 10qter subtelomere, and hybridisation with the 4qA probe identifies genomic sequences from both subtelomeric regions. The 4qA- and 4qB-defined 4qter subtelomeres are found to occur with almost equal frequency in the Caucasian population.[8] However, essentially all FSHD patients exhibit highly biased 4qA/4qB allelic frequencies, in which disease expression is not only associated with large D4Z4 contractions, but that these small (<38kb) D4Z4-containing EcoRI/BlnI fragments must be specifically located on a 4qA-defined 4qter subtelomere.[8][9] Conversely, small EcoRI/BlnI fragments that are located on either a 4qB-defined 4qter subtelomere, or on a 4qA-defined 10qter subtelomere, are either not disease-associated, or they demonstrate a greatly reduced disease penetrance.[10] In a very small number of DNA samples analysed, both from FSHD patients and normal individuals, the small 4q35-derived D4Z4-containing HindIII fragments failed to hybridise to either probe 4qA or 4qB, indicating the possible presence of an additional 4qter subtelomeric type in humans.[11]

This large patient study was therefore undertaken to confirm that it is only large D4Z4 deletions located on a 4qA-defined 4qter subtelomere that are associated with FSHD disease expression. The study involved the analysis of DNA samples from 164 unrelated affected individuals, along with a smaller number of normal individuals. Each of the FSHD patients involved in the study was known to carry a small, disease-associated, 4q35-derived EcoRI/BlnI fragment, as identified by hybridisation to probe p13-E11. Probes 4qA and 4qB were hybridised to HindIII-digested genomic DNA, from patients and controls, separated either by pulsed-field gel electrophoresis (PFGE) or by standard linear gel electrophoresis (LGE), to determine the 4q35-located 4qA / 4qB allele frequencies and to correlate this information with the presence of small, 4q35-located D4Z4 repeat arrays.

MATERIAL AND METHODS

FSHD patients
The FSHD patient cohort contained DNA samples from 164 unrelated affected individuals, either obtained for FSHD research purposes, or from those patients referred to our molecular diagnostic facility for FSHD analysis. The majority of patients were from the UK (144), with 15 patients from Turkey and 5 from Australia. The patients included in the study all conformed to accepted FSHD clinical criteria.

High molecular weight DNA (HMW-DNA) was available for PFGE from 15 Turkish and 50 British FSHD patients, and from 15 Turkish and 35 British normal individuals. Standard DNA, suitable for linear gel electrophoretic (LGE) analysis, was available from a further 99 unrelated FSHD patients, of which 35 were familial disease cases. Control DNA from 50 normal individuals was also available.

All the FSHD patients had previously undergone DNA diagnosis and had been identified as carrying small (~38kb, <11 repeats), 4q35-located D4Z4 repeat arrays, as determined by p13E-11 hybridisation to EcoRI- and EcoRI/BlnI-digested genomic DNA.[12]

DNA isolation, analysis of D4Z4 repeat size, and 4qA and 4qB variant determination

Standard and high molecular weight DNA was isolated from peripheral blood lymphocytes from FSHD patients and controls using previously published methods.[13] For PFGE analysis, 5µg HMW-DNA was used for each restriction digest. The 4qA- and 4qB-defined subtelomeric alleles were identified by the sequential hybridisation of the probes p4qA and p4qB to HindIII-digested DNA separated on a 0.4-0.5% agarose gel electrophorised at 28V for 72 to 48 hours and blotted onto Hybond membrane.[14]

RESULTS

All 164 unrelated FSHD patients were previously identified as having small (<38kb) 4q35-located EcoRI/BlnI fragments. No evidence for somatic mosaicism involving this small EcoRI/BlnI fragment was found in any of the unaffected parents of affected sporadic FSHD patients who were fully investigated. In many of these de novo cases only the patient's DNA was available for a complete screen with p13-E11, and the 4qA and 4qB probes. Xap1 digests of the DNA samples were not routinely carried out, precluding determination of 10qA allele frequencies.

DNA samples from normal individuals, of both Turkish and UK origin, were all found to display equivalent frequencies for the 4q35-located 4qA and 4qB markers, with 53% 4qA and 47% 4qB alleles found overall. The study also demonstrated that either HindIII-digested HMW-DNA analysed by PFGE, or HindIII-digested standard DNA analysed by LGE, was equally proficient in resolving 4qA- and 4qB-specific bands. Whilst PFGE-based analysis is found to give greater accuracy when sizing the several DNA restriction fragments, the much greater speed, and the decreased technician time required when this analysis is carried using standard DNA and LGE, has made this the system of choice in our routine molecular diagnostic laboratory.

An example of the hybridisation results for probe p13-E11 to EcoRI and EcoRI/BlnI-digested DNA, and probes p4qA and p4qB to HindIII-digested DNA from a typical FSHD patient is shown in Figure 2. PFGE-based analysis of 65 unrelated FSHD patients, 15 from Turkey and 50 from the UK, demonstrated the complete concurrence of a small (<11 repeats) D4Z4 array located on 4qA-defined 4q35-located subtelomere in each patient. While analysis of the normal chromosome 4 in each of these 65 patients demonstrated an almost equal frequency for the 4qA (54.5%) and 4qB (45.5%) alleles. In the more extensive LGE-based analysis of HindIII-digested standard DNA from 99 unrelated FSHD patients, it was found that 97 of these samples exhibited a small 4q35-located 4qA allele compatible with their small (~38kb, <11 repeats), EcoRI/BlnI fragment. In the DNA from the two remaining FSHD patients, however, neither probe 4qA nor 4qB hybridised to any small 4q35-specific DNA fragments,
even though the expected number of non-specific gel bands were observed with both probes. The hybridisation results from applying probes p13-E11, p4qA and p4qB to EcoRI/BlnI- and HindIII-digested DNA from one of these FSHD patients is shown in Figure 3.

**DISCUSSION**

Assessment of 4qA and 4qB allele frequencies in a large cohort of 164 unrelated FSHD patients, that included 15 unrelated Turkish patients, confirmed the almost exclusive association between FSHD disease-expression and the presence of small (~38kb, <11 repeats) D4Z4 repeat arrays located on small 4qA-defined 4qter subtelomeres. The FSHD patient sample surveyed more than doubles the numbers reported in two previous similar studies.[8][9] As none of the normal individuals analysed demonstrated a small (<38kb) 4q35-located D4Z4 repeat array, we were unable to corroborate whether any of these were associated with a small 4qB-defined 4qter subtelomere, and would, therefore, be considered to be non-disease-associated.[10]

The 4q35-located 4qA / 4qB allele frequencies determined in the 15 normal Turkish DNA samples demonstrated an equal prevalency for 4qA- and 4qB-defined 4qter subtelomeres, demonstrating no apparent difference in the 4qA/4qB allele frequencies in this ethnic subpopulation.

An additional rare type of 4qter subtelomere was identified following the repeated failure of hybridisation both probes 4qA and 4qB to any small 4q35-specific DNA fragments from two FSHD patients, known to have small (24kb and 21kb) EcoRI/BlnI fragments. None of the normal individuals analysed gave evidence for an alternative 4qter subtelomere, although this has been reported.[11] It is not known if there is any relationship between this rare 4qter subtype and FSHD disease expression.

This large FSHD patient study has corroborated and strengthened the evidence for the almost exclusive association between disease expression and the location of the FSHD locus on a small 4qA-defined 4qter subtelomere. Confirmation of this essentially complete allelic association greatly increases confidence in implementing the use of the 4qA and 4qB probes in routine molecular diagnoses of FSHD. This maybe especially important in those FSHD cases which demonstrate extreme variability in their clinical presentation.

**ACKNOWLEDGEMENTS**

We thank all the clinicians who have provided us with FSHD patient samples, especially Dr Peter Lunt (Bristol), Dr Mark Rogers (Cardiff), and Dr Piraye Serdaroglu (Turkey). We gratefully acknowledge the financial support of the Association Française contre les Myopathies (AFM). Finally, we must ever express our continuing thanks to the many FSHD patients, and their families, who have for many years given us their full support and encouragement.

**COMPETING INTERESTS** No financial interests declared by the authors

**LICENCE FOR PUBLICATION**
The corresponding author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd to permit this article (if accepted) to be published in Journal of Medical Genetics and any other BMJPG products and sub-licences such use and exploit all subsidiary rights, as set out in our licence (http://JMG.bmjjournals.com/misc/ifora/licenceform.shtml).

FIGURE LEGENDS

Figure 1: Genomic map of the FSHD locus region containing 4qA- and 4qB-defined 4qter subtelomeres

A schematic map of the 4qter subtelomeric region. The location of the highly variable D4Z4 repeat array, the position of the p13-E11, p4qA and p4qB-defined sequences, the location of the relevant restriction enzyme sites, and the position of the ~8.0kb beta-satellite sequence that defines the 4qA-containing 4qter subtelomere are illustrated. The large D4Z4-containing HindIII fragment identified by p4qA and p4qB hybridisation is also shown.

Figure 2: PFGE gel analysis of FSHD patient DNA.

EcoRI and EcoRI/BlnI DNA digests hybridised to p13-E11 (track 1 & 2). HindIII DNA digests hybridised to probe 4qA (track 3) and probe 4qB (track 4)

Chromosome 4-specific bands (▲) and chromosome 10-specific bands (★) are indicated. All non-specific hybridising bands are identified (ｾ). The HindIII-digested DNA hybridises only to probe 4qA (track 3) and not to probe 4qB, indicating that this FSHD patient has an AA genotype at 4q35.

Figure 3: Analysis of DNA from a FSHD patient lacking 4qA and 4qB alleles.

Track 1: DNA from patient digested with EcoRI/BlnI hybridised with p13-E11 a 4q35-derived 24kb band is indicated (★).

Tracks 2 / 3: Patient DNA digested with HindIII and hybridised to probe 4qA [2] and probe 4qB [3]. No band comparable to the 24kb EcoRI/BlnI was found, an ~30kb 4qA band would be expected (within the region of the gel marked {). Only non-specific hybridising bands are present (ｾ).

REFERENCES

1 Padberg GW. Facioscapulohumeral disease. Thesis. Leiden University, Leiden,
The Netherlands 1982.


8 Lemmers RJ, Kievit P de, Sandkuijl L, Padberg GW, Ommen GJ van, Frants RR, Maarel SM van der. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere. *Nat Genet* 2002;32:235–236.


Figure 1