Clinical features of facioscapulohumeral muscular dystrophy 2

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ABSTRACT

Objective: In some 5% of patients with facioscapulohumeral muscular dystrophy (FSHD), no D4Z4 repeat contraction on chromosome 4q35 is observed. Such patients, termed patients with FSHD2, show loss of DNA methylation and heterochromatin markers at the D4Z4 repeat that are similar to patients with D4Z4 contractions (FSHD1). This commonality suggests that a change in D4Z4 chromatin structure unifies FSHD1 and FSHD2. The aim of our study was to critically evaluate the clinical features in patients with FSHD2 in order to establish whether these patients are phenotypically identical to FSHD1 and to establish the effects of the (epi-) genotype on the phenotype.

Methods: This cross-sectional study studied 33 patients with FSHD2 from 27 families, the largest cohort described to date. All patients were clinically assessed using a standardized clinical evaluation form. Genotype analysis was performed by pulsed field gel electrophoresis and PCR; D4Z4 methylation was studied by methylation-sensitive Southern blot analysis.

Results: FSHD2 is identical to FSHD1 in its clinical presentation. Notable differences include a higher incidence (67%) of sporadic cases and the absence of gender differences in disease severity in FSHD2. Overall, average disease severity in FSHD2 was similar to that reported in FSHD1 and was not influenced by D4Z4 repeat size. In FSHD2, a small effect of the degree of hypomethylation on disease severity was observed.

Conclusions: Clinically, patients with FSHD2 are indistinguishable from patients with FSHD1. The present data suggest that FSHD1 and FSHD2 are the result of the same pathophysiologic process. *Neurology*® 2010;75:1548–1554

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GLOSSARY

CSS = clinical severity score; **FSHD** = facioscapulohumeral muscular dystrophy; **PBL** = peripheral blood lymphocyte; **SSLP** = simple sequence length polymorphism.

Patients with facioscapulohumeral muscular dystrophy (FSHD) present with a characteristic pattern of muscle weakness with a descending progression starting in the face and scapular fixators, then progressing to humeral muscles, foot dorsiflexors, and finally, proximal lower extremity muscles.[1] Other features include striking side-to-side asymmetry, abdominal muscle weakness, and rare extramuscular manifestations.[2,3]

In most patients with FSHD (FSHD1), the genetic lesion consists of a contraction of the macrosatellite repeat array D4Z4 in the subtelomere of chromosome 4q to <11 repeat units occurring on a specific 4qter genetic background, the 4A161 haplotype.[4-6]

Supplemental data at www.neurology.org

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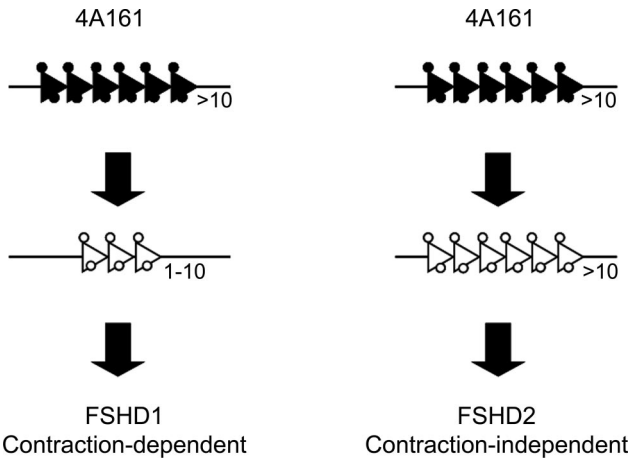
Figure 1 **Two conditions predispose to facioscapulohumeral muscular dystrophy (FSHD)**



Both contraction-dependent and contraction-independent D4Z4 chromatin changes on the permissive 4A161 haplotype result in FSHD development. Black triangles and black dots represent a more closed chromatin structure as found in control individuals. White triangles and white dots correspond to a more open chromatin structure as found in patients with FSHD1 and patients with FSHD2.

The contracted D4Z4 repeat in FSHD1 undergoes considerable changes in its chromatin structure, including loss of DNA methylation[7] and loss of the histone modification H3K9me3.[8] Similar chromatin changes are also observed in a minority of patients with clinical FSHD (FSHD2) lacking a D4Z4 contraction.[7-9] Unlike FSHD1, patients with FSHD2 show loss of DNA methylation on both chromosome 4q and 10q D4Z4 repeats, suggesting that a defect in establishing or maintaining the D4Z4 repeat chromatin structure underlies FSHD2.[10] We postulate that either contraction-dependent or contraction-independent chromatin changes on the permissive 4A161 haplotype result in the development of FSHD (figure 1).

If both FSHD1 and FSHD2 result from an identical pathophysiologic cascade triggered by chromatin changes of 4q D4Z4 repeats, we predict that FSHD2 is phenotypically identical to FSHD1. In the present study we critically evaluate the clinical features of FSHD2, correlate them to the patients' genetic and epigenetic status, and determine if the more pronounced and widespread D4Z4 hypomethylation influences disease severity.

METHODS

Patients and controls. Study subjects were recruited from academic institutions in 8 countries. All were analyzed for their clinical features, D4Z4 repeat size and constitution, proximal simple sequence length polymorphism (SSLP), and distal A/B variation on chromosomes 4q and 10q. After informed consent was obtained, DNA samples derived from peripheral blood lymphocytes (PBLs) of 27 FSHD2 families were collected (figure e-1 on the *Neurology*® Web site at www.neurology.org). In addition, DNA samples of independent patients with FSHD1 and control individuals were included for comparison.

Standard protocol approvals and patient consents. The study protocol was approved by the institutional review boards at the Leiden University Medical Center, The Radboud University Nijmegen Medical Centre, and the University of Rochester Medical Center. In addition, written informed consent was obtained from all patients and unaffected relatives participating in the study.

Clinical assessment. Clinical data on patients with FSHD2 were collected using standardized forms that ensured that identical data points were collected on the study subjects across the multiple sites (data form available online: http://www.urmc.rochester.edu/fields-center/protocols/). Clinical assessments included 1) patient-estimated age at symptom onset, 2) patient-reported initial symptoms or signs, 3) family history of FSHD, 4) history of retinal vascular problems or hearing loss, 5) neuromuscular examination, 6) scoring of the clinical severity score (CSS),[11] and 7) diagnostic category based on previously published clinical criteria for FSHD.[12]

SSLP, repeat length, and distal variation determination. A simple sequence length polymorphism (SSLP) is located 3.5 kb proximal to D4Z4 that is polymorphic for the different 4q and 10q haplotypes. This SSLP was studied by PCR as described previously.[6] For D4Z4 repeat sizing and determination of the distal A/B variation, agarose plugs (InCert agarose [FMC]) containing genomic DNA isolated from 5×10^5 PBLs per plug were digested according to manufacturer's instructions with combinations of *Bln*I (Takara), *Eco*RI, *Hind*III, and *Xap*I (MBI Fermentas).[13] Next, DNA was separated on a 0.85% agarose gel (MP agarose [Roche]) by pulsed field gel electrophoresis and transferred to a hybond-XL membrane (GE Healthcare) by Southern blotting.[6] The resulting membranes were hybridized overnight with the ^{32}P-labeled probe p13E-11[4] for D4Z4 sizing or sequentially with the ^{32}P-labeled probes 4qA or 4qB[13] for A/B typing. After washing in 2 × SSC/0.1% SDS (D4Z4 sizing), 1 × SSC/0.1% SDS (4qA), or 0.3 × SSC/0.1% SDS (4qB), the membranes were exposed to a PhosphorImager screen.

D4Z4 methylation. D4Z4 methylation levels were determined in the proximal D4Z4 repeat units of chromosomes 4q and 10q using the (methylation-sensitive) restriction enzymes *Bln*I (Takara), *Bgl*II, *Cpo*I, *Eco*91I, *Eco*RI, *Xap*I (MBI Fermentas), *Bsa*AI, and *Fse*I (New England BioLabs). The methods applied were described before.[7,10] Briefly, 4 μg of genomic DNA was digested overnight with a combination of (methylation-sensitive) restriction enzymes followed by linear gel electrophoresis, Southern blotting, and hybridization with the ^{32}P-labeled probe p13E-11.[4] After washing with SSC/SDS buffers with diminishing gradient and exposure to a PhosphorImager screen, signal intensities were quantified with ImageQuant software (Molecular Dynamics).

Statistical analysis. The size of the shortest 4A161 repeat, the D4Z4 methylation levels, and the age-corrected CSS were statistically evaluated between patients with FSHD1 and patients with FSHD2 using either independent Student *t* tests or Mann-

Whitney *U* test. These same tests were used to compare age-corrected CSS between patients with FSHD2 with a single 4A161 chromosome or 2 4A161 chromosomes and to compare age-corrected CSS between female and male patients with FSHD2. The Pearson χ^2 test was performed to compare the frequencies of 4q and 10q haplotypes between patients with FSHD1 and patients with FSHD2. Correlations between age-corrected CSS and D4Z4 repeat size or D4Z4 methylation levels were determined using Pearson correlation coefficient. All statistical analyses were performed using SPSS software (version 16.0).

RESULTS Patients were assigned as FSHD2 when the following criteria were met: 1) a phenotype consistent with FSHD, 2) no D4Z4 repeat <11 units present on the permissive 4A161 haplotype, and 3) low D4Z4 methylation levels on chromosomes 4q and 10q. As a threshold for D4Z4 hypomethylation, upper limits were determined on the basis of average methylation levels at identical restriction sites in at least 50 independent control individuals (figure e-2).

Clinical characteristics. Of the 33 patients with FSHD2, 20 (61%) were male. The average age at symptom onset was 26 years (range 0–60), which is almost 10 years later than in FSHD1.[1] The initial symptom was scapular weakness in 61%, foot dorsiflexor weakness in 27%, facial weakness in 10%, and hip girdle weakness in 3%. On examination, all had scapular weakness and 79% had foot dorsiflexor weakness and all but 2 patients had facial weakness. However, both patients without facial weakness had typical scapular as well as ankle dorsiflexor weakness and one had a relative with a typical FSHD phenotype including facial weakness. A positive Beevor sign, said to be specific for FSHD,[2,14] was found in 12/18 patients tested (67%). Ten patients also had pronounced asymmetry in their limb weakness. The average CSS on the modified Ricci scale was 6 (±2) (range 1–10) for the cohort, similar to that reported in a study of 65 patients with FSHD1.[15] The average age-adjusted CSS (144 ± 48) was not different between males and females ($p = 0.632$). Three patients (9%) were nonambulatory. Symptomatic hearing loss was reported in 6 (18%) patients and none reported retinal vascular problems. Yet a systematic evaluation of the retinal vessels was not performed as part of the study except in 2 patients who demonstrated no retinal vasculopathy on indirect ophthalmoscopy.

One clear distinction between FSHD1 and FSHD2 is the mode of inheritance. The majority (20/33) were sporadic, 11 were familial, and in 2 the inheritance pattern was uncertain. Therefore, the familial to sporadic ratio in FSHD2 is inverse to the ratio in FSHD1. Of the definite familial cases, 3 seem dominant in inheritance (parent-child pairs) and 2 seem recessive in inheritance (sibling pairs).

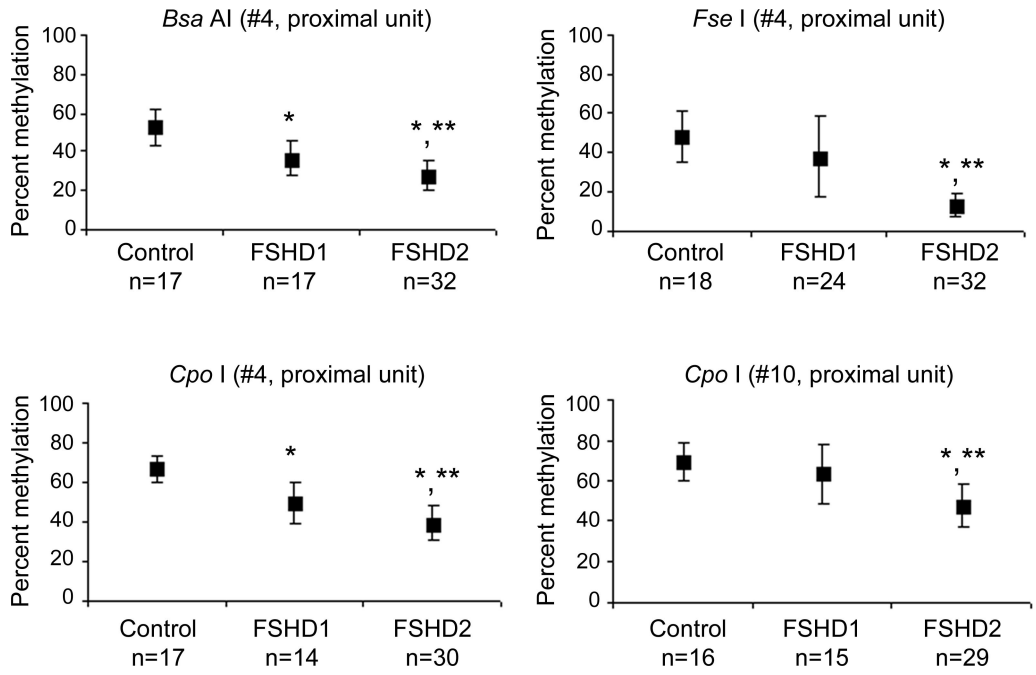
Genotyping and D4Z4 methylation. The genotypes of patients with FSHD2 are shown in table e-1 and are summarized in table 1. Additionally, genotyping re-

Table 1 Genotyping results of the simple sequence length polymorphism proximal to D4Z4, the repeat sizing, and the determination of the distal A/B variation in control individuals, patients with FSHD1, and patients with FSHD2[a]

	Control (n = 50), %	FSHD1 (n = 50), %	FSHD2 (n = 33), %
Shortest 4A161 repeat	28 units ± 13	6 units ± 2	16 units ± 8
4A161 haplotype	36.0	63.0	72.7
4A163 haplotype	1.0	0.0	0.0
4A166 haplotype	1.0	2.0	4.5
4A166[b] haplotype	8.0	3.0	0.0
4B161 haplotype	0.0	0.0	1.5
4B162 haplotype	3.0	2.0	1.5
4B163 haplotype	35.0	21.0	15.3
4B168 haplotype	16.0	9.0	4.5
10A164 haplotype	6.0	4.0	1.5
10A166 haplotype	86.0	86.0	84.8
10A176[c] haplotype	1.0	6.0	6.1
10B161[c] haplotype	7.0	4.0	7.6
Short repeat on non-4A161	12.2	18.2	10.6

Abbreviation: FSHD = facioscapulohumeral muscular dystrophy.
[a] The average (±SD) D4Z4 array size of the shortest 4A161 repeat, the frequencies of the different 4q and 10q haplotypes, and the frequency of short repeats on a non-4A161 haplotype are shown.
[b] 10q-type D4Z4 repeat on chromosome 4.
[c] 4q-type D4Z4 repeat on chromosome 10.

Figure 2 D4Z4 methylation levels in control individuals, patients with facioscapulohumeral muscular dystrophy (FSHD) 1, and patients with FSHD2



D4Z4 methylation levels were determined using methylation-sensitive restriction enzymes in combination with Southern blot analysis. Results are shown for the *Bsa*AI, *Fse*I, and *Cpo*I restriction sites in the proximal D4Z4 repeat unit on chromosome 4q and for the *Cpo*I restriction site in the proximal D4Z4 repeat unit on chromosome 10q. *$p < 0.01$ vs control individuals; **$p < 0.01$ vs patients with FSHD1.

sults for 50 control individuals and 50 independent patients with FSHD1 are summarized in table 1. The results corroborate a previous small study of FSHD2 families[10] and show that all patients with FSHD2 carry at least 1 D4Z4 repeat on the permissive haplotype 4A161. On average, the shortest 4A161 repeat in patients with FSHD2 is 16 units and this is shorter than the average of 28 units observed in 50 control individuals ($p < 0.001$) (table 1).

The frequencies of the different 4q and 10q haplotypes in control individuals, patients with FSHD1, and patients with FSHD2 are shown in table 1. The distribution of the haplotypes of the second chromosome 4q ($p \geq 0.156$) and the distribution of the haplotypes on both chromosomes 10q ($p \geq 0.319$) are not different between the FSHD groups. Thus, patients with FSHD2 do not carry a specific haplotype more often than patients with FSHD1. Furthermore, no difference in the frequency of short repeats on non-4A161 haplotypes between patients with FSHD1 and patients with FSHD2 was found (table 1). Except for the D4Z4 contraction on the permissive 4A161 haplotype, no significant difference at the genotype level is observed between patients with FSHD1 and patients with FSHD2.

The DNA methylation levels of the D4Z4 repeat at 4 methylation-sensitive restriction sites in patients with FSHD2 and their unaffected relatives is reported in table e-1. Additionally, the average D4Z4 methylation levels in patients with FSHD2 were compared with those in at least 14 control individuals and patients with FSHD1. At the 4 sites tested, significant D4Z4 hypomethylation compared with control individuals and similar D4Z4 methylation levels as observed in patients with FSHD1 were detected in patients with FSHD2 (figure 2). These results are fully supported by a previous study of D4Z4 hypomethylation in FSHD.[7] Patients with FSHD2 show significant D4Z4 hypomethylation on chromosomes 4q and 10q, while in patients with FSHD1 significant loss of D4Z4 methylation is restricted to the contracted chromosome 4q.[10]

(Epi-)genotype–phenotype effects. The more pronounced and widespread D4Z4 hypomethylation in patients with FSHD2 did not affect severity. Our FSHD2 cohort had a similar average CSS to that of a published FSHD1 cohort.[15] These results suggest that D4Z4 hypomethylation at the second chromosome 4q and both chromosomes 10q does not influence the phenotype.

In FSHD1 the smallest residual repeat sizes are often found in patients with more severe phenotypes.[11,16,17] In FSHD2 4A161 D4Z4 repeat size did not seem to affect age-corrected CSS ($r = 0.188$, $p = 0.295$).

Additionally, in FSHD1 only severely affected patients with very small residual repeat sizes show very low D4Z4 methylation levels, while patients with arrays between 4 and 7 units show large interindividual variation in both CSS and D4Z4 hypomethylation.[18] Also in our FSHD2 cohort, lower D4Z4 methylation levels at *Bsa*AI ($r = -0.367$, $p = 0.035$) and *Cpo*I sites ($r = -0.428$, $p = 0.018$) were observed in patients with a higher age-adjusted CSS, but not at the *Fse*I site ($r = -0.344$, $p = 0.054$). These findings suggest that the degree of hypomethylation plays a small role in disease severity. However, these results need to be interpreted cautiously and will need corroboration in a larger cohort as exclusion of a single outlier from the dataset changes the p values substantially.

In 2 FSHD1 families with compound heterozygotes for 2 FSHD-sized alleles, a possible phenotypic dosage effect was observed but a more recent study failed to show a more severe phenotype in a patient carrying 2 identical FSHD-sized alleles.[19,20] Fifteen patients with FSHD2 in our collection carried 2 hypomethylated 4A161 chromosomes. However, these patients were not more severely affected than patients with a single 4A161 chromosome ($p = 0.664$).

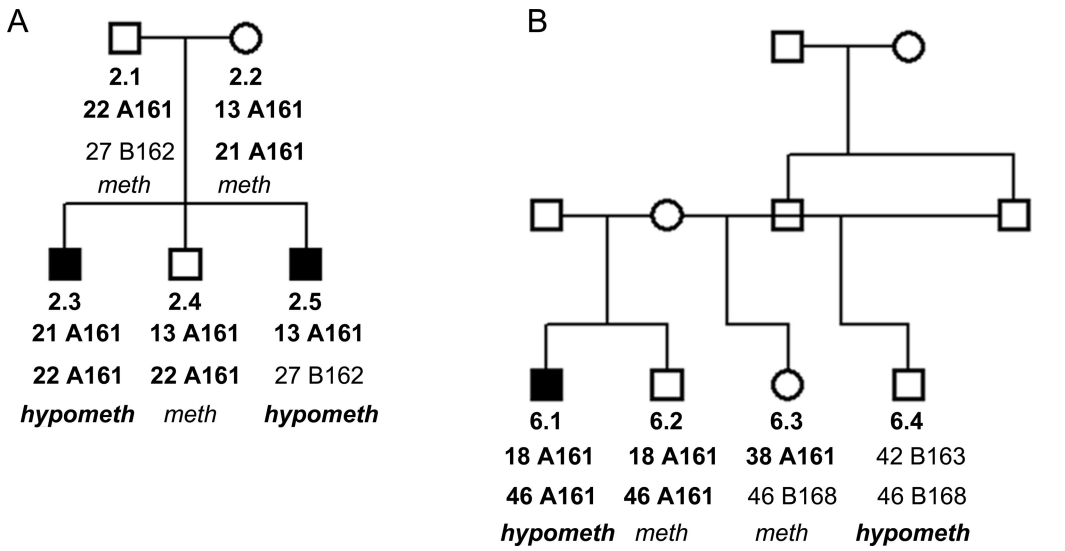
Two FSHD2 families studied in detail. Family 2 (figure 3A) includes 2 brothers with FSHD2. Although both brothers present with D4Z4 hypomethylation, they carry 4A161 chromosomes with different repeat sizes. Apparently, for the development of FSHD2 D4Z4 hypomethylation and a 4A161 chromosome, but not a specific 4A161 chromosome, are essential. Similar findings are observed in FSHD2 families 20 and 26 (table e-1).

Family 6 (figure 3B) consists of 4 siblings; the oldest has FSHD2. The second son carries the same D4Z4 chromosomes as his affected brother but does not have FSHD2 as he has normal D4Z4 methylation levels. The youngest unaffected son, who is 29 years old, shows D4Z4 hypomethylation but lacks the permissive 4A161 haplotype. Thus, this family shows that unaffected relatives may present with D4Z4 hypomethylation at chromosomes 4q and 10q in the absence of a permissive 4A161 chromosome.

DISCUSSION This study of 33 patients with FSHD2, the largest cohort to date, critically assessed the FSHD2 phenotype compared to that of FSHD1 and determined the effect of the specific genotype and methylation patterns on the clinical manifestations.

The clinical characteristics of our cohort of patients with FSHD2 were identical to FSHD1 in almost all aspects. In fact, all the patients in the cohort, given their typical presentation, were assumed to be FSHD1 and the possibility of FSHD2 was only raised when routine genetic testing was negative. The

Figure 3 Two facioscapulohumeral muscular dystrophy (FSHD) 2 families



(A) Family 2, a Canadian family, consists of parents with 3 sons. Two of them, the oldest and youngest, are affected by FSHD2. Both show D4Z4 hypomethylation and carry the permissive 4A161 haplotype. However, while the oldest brother carries a maternally inherited 4A161 repeat of 21 units and a paternally inherited 4A161 repeat of 22 units, the youngest brother carries a maternal repeat of 13 units on a 4A161 chromosome. Thus, for the development of FSHD2 D4Z4 hypomethylation and a 4A161 chromosome, but not a specific 4A161 chromosome, are necessary. (B) Family 6, a French family, consists of 4 siblings. Only the oldest brother is affected; on both chromosomes 4q the permissive 4A161 haplotype is present and D4Z4 hypomethylation is observed at 3 of the 4 sites tested. Importantly, one of his unaffected brothers carries similar D4Z4 chromosomes but does not present with D4Z4 hypomethylation, while his other unaffected brother has D4Z4 hypomethylation but lacks the permissive 4A161 haplotype present in all patients with FSHD.

pattern of skeletal muscle weakness with initial onset of facial and scapular weakness, often with notable side-to-side asymmetry, in FSHD2 was identical to FSHD1. Abdominal muscle weakness resulting in a Beevor sign, a highly specific sign in FSHD1, was present in most patients with FSHD2 tested.[2,14] As for rare FSHD-specific extramuscular manifestations, 6 patients in our cohort had symptomatic hearing loss. None had symptomatic retinal vasculopathy; this is not surprising given the size of the cohort and the infrequent occurrence of symptomatic retinal vasculopathy. Systematic retinal examination with fluorescence angiography is required to assess whether this extramuscular manifestation also occurs in FSHD2.

As a group, the present FSHD2 cohort had an average disease severity similar to that reported in an FSHD1 cohort.[15] In some FSHD1 reports, women were found to be less severely affected than men.[21,22] In our cohort of clinically affected FSHD2 subjects, there were no gender differences in disease severity. However, 3 unaffected relatives of patients with FSHD2, all female, had genotypes and methylation profiles suggesting that they are asymptomatic nonpenetrant gene carriers (see next paragraph). These findings suggest that female FSHD2 gene carriers are less severely affected. A more systematic genotyping of all at-risk individuals within familial cases is needed to confirm this finding. Another difference between FSHD1 and FSHD2 was the age at symptom onset, nearly a decade later in FSHD2.[1] This may suggest that FSHD2 is milder but more likely it is due to the high incidence of sporadic cases. In dominant FSHD1, the vigilance of affected parents leads to earlier recognition of symptoms in affected offspring.

The overall striking similarity in the clinical presentation of our FSHD2 cohort to FSHD1 may be in part due to ascertainment bias. Screening for FSHD2 in patients with undefined dystrophies may reveal a wider clinical spectrum in FSHD2 than is reflected in our cohort.

The genotyping data from this cohort reinforce an earlier FSHD2 study demonstrating the co-occurrence of hypomethylation on 4q and 10q D4Z4 repeats on the background of at least 1 permissive 4A161 chromosome.[10] As demonstrated by 3 unaffected relatives (6.4, 7.2, and 13.10), D4Z4 hypomethylation occurring on a background other than the permissive 4A161 haplotype is not associated with FSHD2. However, 3 other relatives (12.2, 16.2, and 16.5) carry the 4A161 haplotype and are hypomethylated but do not show signs of FSHD. There may be several explanations for this. First, these female individuals may be regarded as asymptomatic nonpenetrant gene carriers. In FSHD1, approximately 20% of gene carriers either never present with symptoms or become symptomatic at a later age.[23,24] Second, unaffected relatives who show D4Z4 hypomethylation may carry a relatively large D4Z4 repeat on the permissive 4A161 chromosome (12.2 carries a 4A161 D4Z4 repeat of 49 units, 16.2 and 16.5 carry a 4A161 D4Z4 repeat of 27 units). It is indeed remarkable that most patients with FSHD2 present with a medium-sized 4A161 D4Z4 repeat, an observation for which we currently have no explanation.

Our data show that the genotype (D4Z4 repeat size) does not influence disease severity in FSHD2. Additionally, the presence of more widespread hypomethylation does not make patients with FSHD2 more severely affected than patients with FSHD1. However, the data raise the possibility of a small effect of the degree of hypomethylation on disease severity in patients with FSHD2 but this finding needs further corroboration in a larger cohort.

The mode of transmission in FSHD2 remains unclear. Twenty of the 33 patients were sporadic. One explanation for this phenomenon is that FSHD2 development results from 2 independent events: 1) D4Z4 hypomethylation by an as yet unknown mechanism and 2) the presence of at least 1 repeat on the permissive 4A161 haplotype. The limited numbers of cases with familial FSHD2 show both dominant inheritance (affected parent-child pairs) and recessive inheritance (affected sibling pairs lacking an affected parent). It will be important to study additional relatives and to determine the mode of inheritance of D4Z4 hypomethylation. An important family to determine this is family 6; although all 4 siblings have the same mother, 3 different fathers are involved (figure 3B).

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Dr. Jessica C. de Greef.

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DISCLOSURE

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