CLIA Laboratory Testing for Facioscapulohumeral Dystrophy

A Retrospective Analysis

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Abstract

Objective

To summarize facioscapulohumeral muscular dystrophy (FSHD) diagnostic testing results from the University of Iowa Molecular Pathology Laboratory.

Methods

All FSHD tests performed in the diagnostic laboratory from January 2015 to July 2019 were retrospectively reviewed. Testing was by restriction enzyme digestion and Southern blot analysis with sequencing of *SMCHD1*, if indicated. Cases were classified as FSHD1 (4q35 EcoRI size ≤ 40 kb; 1–10 D4Z4 repeats), FSHD2 (permissive 4q35A allele, D4Z4 hypomethylation, and pathogenic *SMCHD1* variant), or non-FSHD1,2. We also noted cases with borderline EcoRI fragment size (41–43 kb; 11 D4Z4 repeats), cases that meet criteria for both FSHD1 and FSHD2, somatic mosaicism, and cases with hybrid alleles that add complexity to test interpretation.

Results

Of the 1,594 patients with FSHD tests included in the analysis, 703 (44.1%) were diagnosed with FSHD. Among these positive tests, 664 (94.5%) met criteria for FSHD1 and 39 (5.5%) met criteria for FSHD2. Of all 1,594 cases, 20 (1.3%) had a 4q35 allele of borderline size, 23 (1.5%) were somatic mosaics, and 328 (20.9%) had undergone translocation events. Considering only cases with at least 1 4q35A allele, D4Z4 repeat number differed significantly among groups: FSHD1 cases median 6.0 (interquartile range [IQR] 4–7) repeats, FSHD2 cases 15.0 (IQR 12–22) repeats, and non-FSHD1,2 cases 28.0 (IQR 19–40) repeats.

Conclusion

FSHD1 accounts for 94.5% of genetically confirmed cases of FSHD. The data show a continuum of D4Z4 repeat numbers with FSHD1 samples having the fewest, FSHD2 an intermediate number, and non-FSHD1,2 the most.

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Glossary

ANOVA = analysis of variance; **CLIA** = Clinical Laboratory Improvement Amendments; **FSHD** = facioscapulohumeral muscular dystrophy; **MC** = molecular combing.

Facioscapulohumeral muscular dystrophy (FSHD) is caused by aberrant transcription of the *DUX4* gene on chromosome 4q35 as a result of chromatin changes including hypomethylation of the D4Z4 repeat array proximal to the gene.^{1–4} D4Z4 arrays in normal individuals have 11–100 repeats (EcoRI fragment size >40 kb).⁵ Patients with FSHD1 have contracted D4Z4 arrays with 1–10 repeats (EcoRI fragment size >40 kb) opening the chromatin structure, resulting in *DUX4* derepression.^{2,6,7} In FSHD2, patients have a pathogenic variant in the *SMCHD1* gene on chromosome 18 and 11 or more D4Z4 repeats; the *SMCHD1* variant leads to D4Z4 hypomethylation and *DUX4* transcription.^{1,2,5,8} Distal to the D4Z4 region are 2 polymorphic variants termed 4qA (with a polyadenylation signal) or 4qB (no polyadenylation signal).^{9,10} Only D4Z4 repeats associated with 4q35A alleles result in FSHD allowing transcription of *DUX4*.^{10,11} Located at 10q26, chromosome 10 contains a homologous region to the D4Z4 repeats on chromosome 4.

The results of FSHD1 and FSHD2 testing have not been reported in a large, clinical laboratory-derived patient sample. Here we analyze the FSHD testing results from a CLIA (Clinical Laboratory Improvement Amendments)–certified diagnostic laboratory in the United States. We describe the relative frequencies of FSHD types 1 and 2 in this dataset including analysis of the genetic variation at the 4q35 locus that adds to the complexity of diagnostic test interpretation. This information will be useful in transitioning to new diagnostic testing platforms and in planning future clinical trials necessary for the development of more effective treatments of FSHD.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

After obtaining University of Iowa institutional review board approval, data from the University of Iowa Molecular Pathology Laboratory were reviewed to identify all FSHD tests performed between January 1, 2015, and July 31, 2019. This time frame was chosen because FSHD2-specific testing (D4Z4 methylation and *SMCHD1* sequencing) was added to the laboratory's diagnostic testing protocols in 2015. All analyses were retrospective.

Genetic Analysis

All genetic testing and analysis were performed in the Molecular Pathology Laboratory at the University of Iowa. Laboratory protocols for FSHD testing were utilized as previously described.^{12,13} Briefly, peripheral blood leukocytes embedded

in agarose plugs were prepared for restriction enzyme digestion and Southern blotting to determine the D4Z4 repeat sizes and A/B haplotypes on chromosomes 4 and 10. Genomic DNA was isolated for Southern blot evaluation of D4Z4 methylation and for next-generation sequencing of *SMCHD1*. For the methylation assay, DNA was initially digested with restriction enzymes EcoRI, XapI, and BglII, followed by a second digestion with the methylation-sensitive enzyme FseI. The extent of testing for each individual sample was dependent on the amount and quality of blood submitted, requisition orders from the patients' physicians, or the results of testing acquired while working through the diagnostic flowchart shown in figure 1.

Cases with 4q35/10q26 translocations were defined by having a skewed ratio, 1 or more hybrid alleles, or both. Skewed ratios were defined by having a total of 4 alleles (4q35-type plus 10q26-type alleles) with a ratio other than 2:2. Hybrid alleles were defined by EcoRI/BlnI restriction fragments more than 3 kb smaller than their EcoRI counterparts or XapI restriction fragments more than 5 kb smaller than their EcoRI counterparts.^{12,14} Cases with somatic mosaicism were identified in Southern blots that showed more than 4 EcoRI fragments with characteristics of 4q35-type alleles or 10q26-type alleles. Table 1 summarizes criteria used to assign genetic diagnoses.

The D4Z4 repeat number was calculated from the EcoRI fragment size using the formula¹²

$$\text{D4Z4 Repeat Size} = \frac{(\text{EcoRI fragment size} - 6.9)}{3.3}$$

Data Collection

All FSHD tests evaluated were collected through the Molecular Pathology Laboratory database. All available data pertaining to the FSHD genetic testing were included. Samples that were repeated were included as 1 case and the results from the separate tests were combined. The geographic location of the referring physicians was collected whenever the information was available in the test requisition.

Statistical Analysis

Descriptive and inferential statistics were used to analyze the data. One-way analysis of variance (ANOVA) and Kruskal-Wallis tests were used to compare methylation values and D4Z4 repeat sizes, respectively.

Data Availability

Anonymized raw data are available upon request from the corresponding author.

Figure 1 Facioscapulohumeral Muscular Dystrophy (FSHD) Diagnostic Workflow

Results

Demographics

A total of 1716 FSHD genetic tests were ordered between January 2015 and July 2019. Of these, 76 tests were excluded from further analysis due to technical difficulties or test cancellations from ordering physicians. An additional 46 tests were excluded due to incomplete testing preventing a definitive diagnostic interpretation. This resulted in a sample size of 1,594 tests. The referring physician location could be determined from the requisitions for 795 samples (49.9% of the total); of these, 741 (93.2%) were received from institutions within the United States, as summarized in table 2.

Genetic Analysis

Of the 1,594 genetic tests, 703 cases (44.1%) met criteria for a genetic diagnosis of FSHD. Of the total FSHD cases, 664 (94.5%) met criteria for a genetic diagnosis of FSHD1 and 39 cases (5.5%) met criteria for a genetic diagnosis of FSHD2. The number of cases for each diagnostic group is shown in table 1. The D4Z4 repeat size and other characteristics of FSHD1, FSHD2, and non-FSHD1,2 patients are summarized in tables 3 and 4.

EcoRI Fragment Size and D4Z4 Repeat Size

We compared the shortest 4q35A allele EcoRI fragment sizes and corresponding calculated D4Z4 repeat numbers for the 3 diagnostic groups (FSHD1, FSHD2, and non-FSHD1,2) (table 3 and figure 2). The calculated D4Z4 repeat sizes of the shortest 4q35A allele for these cases were different from each other (Kruskal-Wallis test; all pairwise comparisons $p < 0.0001$).

Methylation Assay

At the requests of ordering physicians or by following the workflow diagram (figure 1), 550 cases underwent methylation testing. The average methylation values for FSHD1, FSHD2, and non-FSHD1,2 cases were 34.7% ($n = 21$), 13.5% ($n = 39$), and 45.5% ($n = 490$) (figure 3). Of the 550 cases with methylation assays, 98 cases (17.8%) had hypomethylation, defined as $\leq 28\%$. Looking only at those with a 4q35A allele, thus those at risk for FSHD, we found the methylation values among groups (FSHD1, FSHD2, and non-FSHD1,2) to be different (one-way ANOVA; all pairwise comparisons Tukey-adjusted $p < 0.0005$).

SMCHD1 Variants

Based on specific physician request or evidence of hypomethylation (figure 1), 138 cases underwent *SMCHD1* sequencing. Of these, 39 cases met criteria for FSHD2 based on identifying a pathogenic *SMCHD1* variant in addition to hypomethylation and the presence of a permissive 4q35A allele (see criteria in table 1). All pathogenic *SMCHD1* variants are listed in supplemental table 1 (data available from Dryad; doi.org/10.5061/dryad.f1vhhmgv3). An additional 56 cases had D4Z4 hypomethylation, but no *SMCHD1* sequence variant was identified. The average methylation value for the D4Z4 hypomethylation cases without pathogenic *SMCHD1* variants was 22.6%. Of the remaining 43 cases that were sequenced, 24 had borderline low methylation values ($>28\%$, but $\leq 35\%$), 14 had methylation values $>35\%$, and 5 did not undergo methylation testing; in these cases, no pathogenic variants were identified.

Rearrangements and Mosaic Samples

Table 4 summarizes the cases in which translocations or somatic mosaicism were identified. Not all cases underwent

Table 1 Genetic Diagnosis Criteria and Number of Cases for Each Diagnostic Category

Genetic diagnosis	Case assignment criteria	Cases, n
FSHD1	<ul style="list-style-type: none"> • 4q35A allele with EcoRI fragment ≤ 40 kb (1–10 D4Z4 repeats) or • 4q35 allele with EcoRI fragment ≤ 40 kb (1–10 D4Z4 repeats), but haplotyping not requested 	664
Borderline FSHD1	<ul style="list-style-type: none"> • 4q35 allele with EcoRI fragment 41–43 kb (11 D4Z4 repeats) and • Permissive 4q35A allele OR haplotyping not requested • No <i>SMCHD1</i> variant^a 	20
FSHD2	<ul style="list-style-type: none"> • Permissive 4q35A allele and • Hypomethylation and • <i>SMCHD1</i> variant^a 	39
FSHD1 + FSHD2	<ul style="list-style-type: none"> • 4q35A allele with EcoRI fragment 33–43 kb (8–11 D4Z4 repeats) and • Hypomethylation and • <i>SMCHD1</i> variant^a 	4
Non-FSHD1,2 with at least 1 4q35A allele	<ul style="list-style-type: none"> • Fail to meet criteria for FSHD1, borderline FSHD1, or FSHD2 • At least one 4q35A allele 	522
Non-FSHD1,2 without a 4q35A allele	<ul style="list-style-type: none"> • Fail to meet criteria for FSHD1, borderline FSHD1, or FSHD2 • No 4q35A alleles^b 	345
Total cases		1,594

Abbreviation: FSHD = facioscapulohumeral muscular dystrophy.

^a Previously reported pathogenic variant or in silico analysis consistent with pathogenic. Variants are summarized in supplemental table 1 (data available from Dryad).

^b The cases without 4q35A alleles include those where all alleles are 10q26-type alleles, all 4q35 alleles are B haplotype, or all 4q35 alleles are >43 kb, but haplotyping was not requested.

EcoRI fragment sizing (for example, the ordering physician requested only a methylation assay or *SMCHD1* sequencing). However, among the 1,573 cases analyzed by Southern blot, 328 tests (20.9%) had evidence of 4q35/10q26 translocations (skewed 4:10 ratios, hybrid alleles, or both). There were 143 (9.1%) hybrid alleles and 230 (14.6%) skewed ratios. Six of the 143 cases with hybrid alleles also showed somatic mosaicism.

Distribution of A and B Haplotypes Among 4q35 Alleles

The 4q35A and 4q35B allele frequencies were examined in all cases where this information was available. From a total of 2,641 alleles with a haplotype designation, 64.6% ($n = 1704$) were haplotype A and 35.4% ($n = 937$) were haplotype B.

Borderline FSHD1 Cases

Due to the imprecise determination of restriction fragment sizes through Southern blotting, we included a category termed borderline FSHD1. These cases had borderline short EcoRI restriction fragments (41–43 kb) found on 4q35A alleles or did not have haplotyping requested (table 1). These cases were excluded from analysis in all above categories except for when examining rearrangements and somatic mosaics. Twenty cases met our diagnostic criteria for borderline FSHD1 with an average EcoRI fragment size of 42.3 kb (SD 0.47). All cases (by

definition) had 11 D4Z4 repeats. Five of these 20 cases did not undergo methylation or *SMCHD1* testing. The average methylation value of the cases tested was 37.4% ($n = 15$). Five of the borderline cases had a methylation level $\leq 28\%$ resulting in *SMCHD1* sequencing following flowchart guidelines, but none of these 5 cases harbored a pathogenic *SMCHD1* variant.

FSHD1 + FSHD2

While examining the cohort of FSHD2 diagnoses, 4 cases met criteria for both FSHD1 and FSHD2. These cases were excluded from analysis in all the above categories except when examining rearrangements and somatic mosaics. The average EcoRI fragment size for the smallest 4q35A alleles was 40.8 kb (38–42 kb); the average methylation value was 13.3% (10%–15%). The pathogenic *SMCHD1* variants for all 4 cases are included in supplemental table 1 (data available from Dryad; doi.org/10.5061/dryad.flvhmgv3) along with the 39 FSHD2 cases described above.

Discussion

This retrospective analysis describes data from the University of Iowa, an academic institution offering CLIA-certified laboratory testing, for the purpose of reporting the relative

Table 2 Geographic Origins of Tests Received Between January 2015 and July 2019

	N (total = 1,594)
United States	
Northeast ^a	209
Southeast ^a	82
Midwest ^a	261
Southwest ^a	17
West ^a	172
Canada	31
Other international	23
Unknown	799

^a The states assigned to each region are as follows. Washington, DC, is included in the northeast region.

Northeast: Connecticut, Delaware, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont. Southeast: Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, West Virginia. Midwest: Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin. Southwest: Arizona, New Mexico, Oklahoma, Texas. West: Alaska, California, Montana, Nevada.

frequencies of FSHD1 and FSHD2 and other genetic characteristics among individuals with a clinical rationale for undergoing FSHD testing. This is an unbiased sample of FSHD diagnostic testing. The results appear to be representative for the US population, as demonstrated by the geographic distribution shown in table 2. In our sample set of nearly 1,600 patients, a genetic diagnosis of FSHD was reached in 44% of cases. Of these, 94.5% (n = 664) had a genetic diagnosis of

FSHD1 and 5.5% (n = 39) a diagnosis of FSHD2. The D4Z4 repeat arrays were significantly smaller in tests consistent with FSHD1. We compared our data to FSHD registry-derived data that reported the distribution of D4Z4 repeat sizes among 74 patients with a genetic diagnosis of FSHD1.⁴ The Iowa Molecular Pathology Laboratory data summarized in table 3 by allele size is similar to the published data: 1–3 D4Z4 repeats (Iowa 16.4% vs registry 12.2%), 4–6 D4Z4 repeats (Iowa 48.2% vs registry 50%), 7–10 repeats (Iowa 35.4% vs registry 37.8%).

Genetic testing for FSHD has been refined over many years. FSHD was first linked to the 4q35 region in 1990.¹⁵ Two years later, in 1992, clinical genetic testing for FSHD became a possibility with the identification of a deleted 4q35 as the genetic basis for the disease.^{16–18} The deletion was determined to be within a region of 3.3 kb D4Z4 repeats that could be evaluated by Southern blots of EcoRI restriction enzyme digests. However, the interpretation of deleted 4q35 alleles was complicated by the high homology with D4Z4 repeats on chromosome 10 (10q26 locus). The next step in diagnostic refinement was the introduction of a EcoRI/BlnI double digestion step to distinguish the D4Z4 repeats derived from 4q35 and 10q26, as the chromosome 10 D4Z4 repeats have BlnI sites not present in 4q35 D4Z4 repeats.^{19,20} XapI digestion further refined testing by digesting 4q35-derived D4Z4 by not those from chromosome 10.²¹ Sequencing DNA distal to the D4Z4 repeats led to the recognition of “A” and “B” haplotypes; abnormally short D4Z4 repeats on 4q35 without an A haplotype (4q35B alleles) were not associated with FSHD and A/B haplotyping was added to the diagnostic workflow.^{22,23} In 2012, pathogenic variants in the *SMCHD1* gene were found in families with an FSHD phenotype but without a 4q35 deletion.¹ The importance of methylation

Table 3 Genetic Characteristics of Tests With a Molecular Diagnosis of FSHD1, FSHD2, or Non-FSHD1,2

	FSHD1	FSHD2	Non-FSHD1,2 (cases with at least on 4q35A allele)
Total, n	664	39	522
Median D4Z4 size (IQR)^a	6 (4–7)	15 (12–22)	28 (19–40)
1–3 repeats, n (%)	109 (16.4)	0 (0)	0 (0)
4–6 repeats, n (%)	320 (48.2)	0 (0)	0 (0)
7–10 repeats, n (%)	235 (35.4)	0 (0)	0 (0)
11–15 repeats, n (%)	0 (0)	20 (51.3)	87 (16.7)
16–20 repeats, n (%)	0 (0)	7 (17.9)	56 (10.7)
21–25 repeats, n (%)	0 (0)	7 (17.9)	92 (17.6)
26–30 repeats, n (%)	0 (0)	3 (7.7)	57 (10.9)
>30 repeats, n (%)	0 (0)	2 (5.1)	230 (44.1)
Median EcoRI Fragment size, kb (IQR)^a	27 (21–30)	55 (48–80)	100 (70–140)

Abbreviations: FSHD = facioscapulohumeral muscular dystrophy; IQR = interquartile range.

^a From Kruskal-Wallis test; all pairwise comparisons $p < 0.0001$.

Table 4 Genetic Rearrangement Characteristics of Tests With a Molecular Diagnosis of FSHD1, FSHD2, or All Non-FSHD1,2, n (%)

	Total (n = 1,573)	FSHD1 (n = 658)	FSHD2 (n = 39)	All non-FSHD1,2 (n = 852)
All translocations ^a	328 (20.9)	131 (20.0)	7 (17.9)	182 (21.4)
Hybrid alleles ^a	143 (9.1)	43 (6.5)	1 (2.6)	94 (11.0)
Skewed ratios ^a	230 (14.6)	104 (15.8)	6 (15.4)	114 (13.4)

Abbreviation: FSHD = facioscapulohumeral muscular dystrophy.

^a Not all cases underwent Southern blotting or had all alleles recorded. Percentages of cases with translocations are calculated from the total cases (n) that underwent Southern blotting to determine the distribution of 4q35-type and 10q26-type alleles.

status was also recognized at this time. Sequencing of *SMCHD1* was added to diagnostic testing, and analysis of methylation could be used to screen for those at risk for an *SMCHD1* variant or to verify the pathogenicity of variants identified by sequencing the *SMCHD1* gene.²⁴ This evolution resulted in the diagnostic workflow utilized by the Iowa Molecular Pathology Laboratory illustrated in figure 1.

Although the genetic understanding of FSHD has developed and grown over the years, the pathophysiology of FSHD is still not fully understood. The muscle wasting found in FSHD is associated with derepression of a gene distal to the D4Z4 repeat array known as the double homeobox 4 (*DUX4*) retrogene. Derepression of *DUX4* is caused by chromatin relaxation due to either D4Z4 contraction (FSHD1) or hypomethylation due to a pathogenic *SMCHD1* variant (FSHD2).²⁵ *DUX4* codes for

a transcription factor that is normally expressed in small amounts in early embryological development and is found in the testis and pluripotent cells but is silenced in adult somatic tissue.^{7,26} Even small amounts of *DUX4* in postnatal humans is toxic to skeletal muscle and results in apoptosis through a cascade of events including disruption of RNA metabolism and induction of oxidative stress.^{27,28} The pathophysiology of FSHD and *DUX4* has been difficult to study due to the retrogene's transient and sporadic misexpression in cells.²⁷

FSHD genetic testing is complex and one of the factors that complicates it is the high degree of homology between 4q35 and 10q26. D4Z4 repeat sizes at 10q26 that are similar to those of pathologic 4q35 alleles do not result in disease manifestations.²⁰ Thus, distinguishing 4q35 from 10q26 alleles is critical to the accurate interpretation of test results.

Figure 2 EcoRI Fragment Sizes and D4Z4 Repeat Sizes Compared Between Diagnostic Categories

EcoRI restriction fragment (A) and D4Z4 repeat sizes (B) compared among groups. Non-FSHD1,2 sizes overlap with FSHD2 sizes but not FSHD1. No FSHD1 cases had values seen in FSHD2 and non-FSHD1,2 cases. (A) The average FSHD1 EcoRI restriction fragment size is smaller than FSHD2 and non-FSHD1,2. (B) The average FSHD1 D4Z4 repeat number is significantly different from FSHD2 and non-FSHD1,2 tests. The non-FSHD1,2 category includes only those cases with at least one 4q35A allele. From Kruskal-Wallis testing, all pairwise comparisons are significantly different ($p < 0.0001$). FSHD = facioscapulohumeral muscular dystrophy.

Figure 3 Comparison of Methylation Values Between Diagnostic Categories

FSHD1 and FSHD2 had average methylation values of 34.7% (n = 21) and 13.5% (n = 39), respectively. All non-FSHD1,2 tests had an average methylation value of 45.5% (n = 490). FSHD1 had a higher range of methylation values when compared to FSHD2 values. When comparing FSHD1, FSHD2, and non-FSHD1,2 tests with at least one 4q35A allele (thus at risk for FSHD), the values were significantly different (one-way analysis of variance; all pairwise comparisons Tukey-adjusted $p < 0.0005$). FSHD = facioscapulohumeral muscular dystrophy.

The high homology found between 4q35 and 10q26 also increases the likelihood of translocations between the 2 loci. Translocation events between 4q and 10q are fairly common within the normal population and have been reported in about 20% of individuals.^{19,29} Remarkably consistent with earlier published data, we found evidence of translocation in 20.9% of individuals tested at Iowa.

A second factor contributing to complexity of FSHD genetic testing is the presence of somatic mosaicism, first described in FSHD in 1993.^{6,17,30–32} We identified 23 cases with somatic mosaicism; they had 5–7 alleles from 4q35 and 10q26. This mosaicism can partially explain the clinical heterogeneity seen among individuals with FSHD.³³ Individuals who are somatic mosaics often have a milder phenotype or are asymptomatic.^{10,33} Of the 10%–30% of FSHD cases that are de novo, about 50% display mosaicism either in a parent or (less commonly) in the proband.^{10,34}

The homology with chromosome 10q, somatic mosaicism, and additional genetic variations, such as p13E-11 probe binding site deletions, have led some to suggest that FSHD testing is inconclusive in about 20% of cases.^{6,31} In our experience, a genetic diagnosis (FSHD1, FSHD2, FSHD1+FSHD2, or non-FSHD1,2) was reached in 96% of cases that underwent testing (n = 1,640). The testing was incomplete in

2.8% of these cases (n = 46), while testing results were borderline FSHD1 in 1.2% (n = 20).

While a D4Z4 repeat array of ≤ 10 units is consistent with a diagnosis of FSHD1, FSHD2 cases also have a relatively short D4Z4 repeat array. FSHD2 cases typically have a D4Z4 repeat array ranging from 8 to 20 units with the average of 12 units, but longer repeat arrays are reported.³⁵ In our study, 27 FSHD2 cases had 12 to 19 repeats and 12 cases had 21 or more repeats, with the highest being 31. One explanation that has been proposed for an FSHD2 diagnosis with a longer D4Z4 sequence is D4Z4 duplications allowing for *DUX4* derepression.³⁵ However, review of the Southern blots at Iowa revealed that none of the 12 cases with more than 21 repeats had evidence of D4Z4 duplications.

One of the FSHD2 cases in our Iowa testing cohort has a 2.5 mb 18p deletion encompassing the *SMCHD1* gene (supplemental table 1; data available from Dryad, doi.org/10.5061/dryad.f1vhhmgv3). Hemizyosity for *SMCHD1* has previously been shown to result in D4Z4 chromatin relaxation characteristic of FSHD in 2 FSHD2 families with a 1.2 MB 18p deletion encompassing *SMCHD*.³⁶ *SMCHD1* hemizyosity also resulted in D4Z4 hypomethylation in 72 of 82 cases with 18p deletions encompassing the *SMCHD1* gene, and the authors proposed that a permissive 4q35A allele with 11–16 D4Z4 repeats might increase the risk of clinical FSHD in these individuals.³⁶ The single case with an 18p deletion in our study has 18 D4Z4 repeats on a permissive 4q35A allele and a D4Z4 methylation value of 14%.

We identified 4 cases with genetic characteristics of both FSHD1 and FSHD2 (table 1); when included in the total number of FSHD cases, they represent 0.6%. *SMCHD1* was first recognized as a disease modifier in FSHD1 cases that were more severely affected than predicted by their D4Z4 allele size (8–10 repeats).^{25,37} In a more recent report, 7 of 19 patients with FSHD with 9–10 D4Z4 repeats also had a pathogenic *SMCHD1* variant, while none of the patients with shorter repeat arrays did. The authors suggested that the 8–10 D4Z4 unit alleles have reduced penetrance and FSHD types 1 and 2 overlap in this repeat range, creating a disease continuum.^{8,38,39} Our cohort includes 2 cases with this genetic signature and 2 additional cases with 11 D4Z4 repeats and an *SMCHD1* variant (see supplemental table 1; data available from Dryad, doi.org/10.5061/dryad.f1vhhmgv3). Due to the previously discussed inconsistencies of allele sizing by Southern blotting, these latter 2 cases were included in our FSHD1+FSHD2 category (n = 4).

Our results and those of others suggest that *SMCHD1* is not the only gene associated with FSHD2. Although we identified 98 tests with 4q35 hypomethylation and a permissive 4qA allele, only 39 had pathogenic *SMCHD1* variants resulting in a FSHD2 diagnosis (not including the FSHD1+FSHD2 cases). Of the remaining 59 cases, 9 had short *EcoRI* fragments resulting in a FSHD1 diagnosis. The other 50 cases were given non-FSHD1,2 genetic diagnoses in the current analysis (table 1); among these

50 cases were 20 individuals with between 11 and 20 D4Z4 repeats who are perhaps at higher risk for FSHD2. It is possible that some of these cases have a pathogenic variant in a non-protein coding region of the *SMCHD1* gene that is not interrogated in the sequencing.²⁵ Recent research at Leiden University Medical Center shows that intronic variants resulting in new splice sites can be found in about 2% of the FSHD2 population.²⁵ Using this as guideline, one of our hypomethylated cases without a FSHD1 or FSHD2 diagnosis is predicted to carry an intronic variant. Alternatively, pathogenic variants in another gene or genes may affect 4q35 methylation status.⁸ One such gene, *DNMT3B* (DNA methyltransferase 3B), is found on chromosome 20q11.^{2,40} Heterozygous autosomal dominant pathogenic variants of *DNMT3B* have been found in patients with clinical characteristics of FSHD and a relatively short D4Z4 repeat array (9–13 repeats) on a 4q35A allele with hypomethylation but no pathogenic *SMCHD1* variant.² Homozygous pathogenic variants in this gene have been implicated in ICF syndrome (immunodeficiency, centromeric instability, and facial dysmorphism).⁴¹ Another possible FSHD2 gene is *LRIF1* (ligand-dependent nuclear receptor-interacting factor 1). A homozygous duplication variant of *LRIF1* was found in 1 patient with a clinical phenotype of FSHD. The patient had a 4q35A allele with 13 D4Z4 repeats and a D4Z4 methylation of 15%.⁴² Current research involving *DNMT3B*, *LRIF1*, and other possible modifiers of FSHD will contribute to understanding the molecular pathogenesis and may contribute to refined diagnostic testing.

The traditionally accepted and preferred method of FSHD testing relies on Southern blot analysis of restriction enzyme digests separated by pulsed field gel electrophoresis, as was used for the tests in this analysis. Southern blotting is labor-intensive, only estimates restriction fragment size, and requires large amounts of high molecular weight DNA.^{31,32} Other testing methods have been explored to overcome these obstacles. One alternative to Southern blotting is molecular combing (MC), where combed DNA is fluorescently hybridized to regions of the D4Z4 repeat region on both chromosome 4 and chromosome 10.³¹ MC reduces the number of steps required for Southern blotting and is reported to provide resolution of cases termed undecided or borderline or where a genetic cause has not been determined by Southern blot.^{6,31,43} Another alternative to Southern blotting is single-molecule optical mapping. DNA molecules are fluorescently tagged either by nick label DNA repair or direct insertion at sequence specific recognition sites. The DNA is electrophoresed and imaged through BioNano technology allowing for D4Z4 sizing and haplotyping.^{32,44} Similar to MC, single-molecule optical mapping reduces the steps needed to diagnosis FSHD. Finally, bisulfite sequencing to measure the methylation level of the D4Z4 repeat region has been used to distinguish among FSHD1, FSHD2, and normal individuals.⁴⁵ Using a series of sequencing steps, 4qA alleles are identified and the level of methylation is used to assign a diagnostic category.⁴⁵ The University of Iowa Molecular Pathology Laboratory is in the process of transitioning

to optical mapping technology to diagnose FSHD1; optical mapping will be used in combination with methylation assays and *SMCHD1* sequencing to diagnose FSHD2.

This report of a large clinical sample of patients from across the United States who underwent testing for FSHD provides unique insights into the genetic epidemiology of this complex disease. Our results highlight some of the complexity involved in genetic testing that will have to be addressed as clinical laboratories move away from Southern blotting and toward more rapid and less expensive approaches to molecular diagnosis. The rapid growth in understanding the pathophysiology of FSHD in recent years is expected to lead to novel approaches to therapy and the population-based data presented here, showing subtype frequencies, will facilitate the design of future clinical trials.

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Autumn Rieken, MS	Departments of Pediatrics, Neurology, and Pathology, Carver College of Medicine, The University of Iowa, Iowa City	Collected, analyzed, and interpreted data; drafted and revised the manuscript for intellectual content
Aaron D. Bossler, MD, PhD	Department of Pathology, Carver College of Medicine, The University of Iowa, Iowa City	Revised manuscript for intellectual content

Continued

Appendix (continued)

Name	Location	Contribution
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Steven A. Moore, MD, PhD	Department of Pathology, Carver College of Medicine, The University of Iowa, Iowa City	Designed and conceptualized study; drafted and revised the manuscript for intellectual content

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