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The Official Journal of the American Academy of Neurology

AMERICAN ACADEMY OF
NEUROLOGY®

Association of 4qA-Specific Distal D4Z4 Hypomethylation With Disease Severity and Progression in Facioscapulohumeral Muscular Dystrophy

Author(s):

Fuze Zheng, MD[1]; Liangliang Qiu, MD[1,2]; Long Chen, MD[1]; Ying Zheng, MD[1]; Xiaodan Lin, MD[1]; Junjie He, MD[1]; Xin Lin, MD[1,2]; Qifang He, MD[1]; Yuhua Lin, MD[1]; Lin Lin, MD[1]; Lili Wang, MD[1]; Feng Lin, MD,PhD[1,2]; Kang Yang, MD[1]; Minting Lin, MD,PhD[1,2]; Yi Lin, MD,PhD[1,2]; Ying Fu, MD,PhD[1,2]; Ning Wang, MD, PhD[1,2]; Zhiqiang Wang, MD,PhD[1,2]

Corresponding Author:

Zhiqiang Wang, fmuwzq@fjmu.edu.cn

Neurology® Published Ahead of Print articles have been peer reviewed and accepted for publication. This manuscript will be published in its final form after copyediting, page composition, and review of proofs. Errors that could affect the content may be corrected during these processes.

from the journal.

Affiliation Information for All Authors: 1.Department of Neurology and Institute of Neurology of First Affiliated Hospital, Institute of Neuroscience, and Fujian Key Laboratory of Molecular Neurology, Fujian Medical University, Fuzhou 350005, China; 2.Department of Neurology, National Regional Medical Center, Binhai Campus of the First Affiliated Hospital, Fujian Medical University, Fuzhou 350212, China

Equal Author Contribution:

FZZ and LLQ contributed equally to this work as first authors.

Contributions:

Fuze Zheng: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Analysis or interpretation of data

Liangliang Qiu: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design

Long Chen: Major role in the acquisition of data

Ying Zheng: Major role in the acquisition of data

Xiaodan Lin: Major role in the acquisition of data

Junjie He: Major role in the acquisition of data

Xin Lin: Major role in the acquisition of data

Qifang He: Major role in the acquisition of data

Yuhua Lin: Major role in the acquisition of data

Lin Lin: Major role in the acquisition of data

Lili Wang: Major role in the acquisition of data

Feng Lin: Major role in the acquisition of data

Kang Yang: Major role in the acquisition of data

Minting Lin: Major role in the acquisition of data

Yi Lin: Major role in the acquisition of data

Ying Fu: Major role in the acquisition of data

Ning Wang: Major role in the acquisition of data

Zhiqiang Wang: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data

Figure Count:

4

Table Count:

3

Search Terms:

[14] All Clinical Neurology, [91] All Genetics, [176] All Neuromuscular Disease, [185] Muscle disease

Abstract

Objective: To examine whether the regional methylation levels at the most distal D4Z4 repeat units (RU) in the 4qA-permissive haplotype were associated with disease severity and progression in facioscapulohumeral muscular dystrophy type 1 (FSHD1).

Methods: This was a 21-year, retrospective, and observational cohort study conducted at the Fujian Neuromedical Centre (FNMC) in China. Methylation levels of the most distal D4Z4 RU, including 10 CpGs, were assessed in all participants by bisulfite sequencing. FSHD1 patients were stratified into 4 groups based on methylation percentage quartiles, including LM1 (low methylation), LM2 (low to intermediate methylation), LM3 (intermediate to high methylation), and highest methylation levels (HM). Patients received evaluations of motor function focusing on lower extremity (LE) progression at baseline and in follow-ups. FSHD clinical score (CS), age-corrected clinical severity scale (ACSS), and modified Rankin Scale were used to assess motor function.

Results: The methylation levels of the 10 CpGs were significantly lower in all 823 genetically confirmed FSHD1 patients than in 341 healthy controls (HCs). CpG6 methylation levels could distinguish: 1) FSHD1 patients from HCs; 2) symptomatic from asymptomatic/unaffected patients; 3) patients with LE involvement from those without LE involvement, with AUCs (95% CI) of 0.9684 (0.9584–0.9785), 0.7417 (0.6903–0.7931), and 0.6386 (0.5816–0.6956), respectively. CpG6 methylation levels were negatively correlated with both CS ($r = -0.392$) and ACSS ($r = -0.432$), and positively correlated with onset age of first-ever muscle weakness ($r = 0.297$). For the LM1, LM2, LM3, and HM groups, the respective proportions of LE

involvement were 52.9%, 44.2%, 36.9%, and 23.4%; and onset ages of LE involvement were 20, 26.5, 25, and 26.5 years. Cox regression analysis—adjusted for sex, age at examination, D4Z4 RU, and 4qA/B haplotype—showed that the LM1, LM2, and LM3 groups (*i.e.*, groups with lower methylation levels) had a higher risk for independent ambulation loss, with HRs (95% CI) of 3.523 (1.565–7.930), 3.356 (1.458–7.727), and 2.956 (1.245–7.020), respectively.

Conclusion: 4q35 distal D4Z4 hypomethylation is correlated with disease severity and progression to lower extremity involvement.

Introduction

Facioscapulohumeral muscular dystrophy type 1 (FSHD1) is among the most common muscular dystrophies,[1] characterized by initial muscular weakness in the face, shoulder, and upper arms, progressing to the trunk and lower extremities.[2] Ultimately, FSHD1 can lead to disability with loss of independent ambulation[3] or even wheelchair dependence.[4] An American team recently reported the relatively high disability rate of 23.7% in their cohort.[5] In China, 12.0% of genetically confirmed FSHD1 patients will likely lose independent ambulation within 40 years of first onset of muscle weakness; in this large scale population studied by our group, the incidence of wheelchair reached 8.9%.[3,4] The development of disability in FSHD1 may involve three processes, including lower extremity involvement, independent ambulation loss, and wheelchair dependence.

FSHD1 is arguably among the most challenging genetic diseases, in terms of understanding its underlying pathogenic mechanism and developing effective treatments, because it is caused by epigenetic dysregulation due to contraction of D4Z4 macrosatellite repeat units (RU) in a disease-permissive 4qA haplotype.[6] In the general population, the D4Z4 RU number typically ranges from 11 to 100−150 copies, whereas RU number is below the threshold of 10 copies in most patients. D4Z4 microsatellite units are enriched with CpG (73%)[7], and previous studies have proposed that low CpG methylation of D4Z4 sequence can serve as a reliable candidate marker in FSHD diagnosis.[8] In addition, it can explain several clinical features, such as penetrance variability, gender bias in severity, and asymmetric muscle wasting.[9] However, whether the D4Z4 hypomethylation is associated with disease severity and FSHD1 progression has not yet been examined in detail.

In this consecutive study, through analysis of sodium bisulfite sequencing (BSS), we were able to detect

CpG methylation status within the most distal D4Z4 RU in permissive 4qA haplotypes of our previously reported large Chinese FSHD1 cohort.[3,10] We assessed the potential association of distal D4Z4 methylation levels with disease severity and progression to lower extremities in FSHD1, as such an association could help clinicians identify FSHD1 patients at risk for early lower extremity involvement and disability.

Methods

Participants and design

This was a retrospective, observational, and consecutive cohort study to investigate potential associations between distal D4Z4 methylation levels and disease severity and progression in genetically confirmed FSHD patients. All participants were recruited consecutively over 21 years (2001 to 2021) from the Fujian Neuromedical Center (FNMC) in China. FSHD1 was diagnosed according to genetic diagnostic criteria as follows: presenting at least one D4Z4 RU (range 1–10) with a 4qA-specific FSHD1-permissive haplotype that provided a polyadenylation signal (PAS) based on a pulsed-field gel electrophoresis (PFGE)-based Southern blotting assay. Healthy controls were spouses and friends of patients with FSHD1 and carried more than 10 D4Z4 RUs. All healthy controls were recruited from the same institute.

Standard protocol approvals, registrations, and patient consents

This study was approved by the ethics committee for Medical Research of the First Affiliated Hospital of Fujian Medical University. Informed consent was obtained from all participants. The ClinicalTrials.gov identifier is NCT04369209 for this study.

Sodium bisulfite sequencing (BSS)-DNA methylation analysis

To assay the regional methylation status of the most distal D4Z4 RU on the permissive haplotype 4qA in this cohort (823 FSHD1 patients and 341 healthy controls), BSS was implemented with a 4qA-allele-specific FasPAS primer that was exclusive to 4qA and not found in 10qA or 4qB (in cooperation with Genesky Biotechnologies, China). The 4qA BSS assay analyzed 10 CpGs following previous protocols.[11, 12] For

participants carrying 4qA/B haplotypes, the distal D4Z4 methylation level was specifically measured in only one 4qA array; for participants carrying 4qA/A haplotypes, the average methylation level was measured on both 4qA arrays as the final distal D4Z4 methylation level.[12]

To investigate differences in the extent of hypomethylation, all 823 FSHD1 patients were stratified into four groups depending on the quartile for the percentage of CpG6 locus methylation levels. Methylation percentage quartiles were calculated such that CpG6 locus methylation levels were ordered from lowest to highest, with the first quartile (Q1; group 1) containing patients with the low methylation (LM1); the second quartile (Q2; group 2) containing patients with low to intermediate methylation (LM2); the third quartile (Q3, group3) including patients with intermediate to high methylation (LM3); and the fourth quartile (Q4, group 4) comprised of patients with the highest methylation (HM). HM levels were used as a reference for comparing differences in FSHD1 disease progression affected by the degree of hypomethylation in Cox regression analyses.

Outcomes of disease progression

The primary outcome was lower extremity involvement, which was defined based on the following two criteria at baseline: patient's reported symptoms and physical signs with an FSHD clinical severity scale (CSS) score greater than or equal to 3 (CSS ≥ 3.0); and independent ambulation loss, where participants were unable to walk without assistance, which was strictly based on the modified Rankin Scale (mRS) with a grade greater than or equal to 4 (mRS ≥ 4).[3] Wheelchair dependency was defined based as mRS of grade 4–5 and a CSS of 4.5–5 (wheelchair use for most activities or wheelchair bound).[4] Yearly follow-ups were conducted through outpatient service, telephone survey, or remote video conferences performed by the same neurologist (Z.Q.W.).

Clinical assessments of FSHD1 patients were performed by the same examiners at study initiation and yearly follow-ups. Clinical data collected included detailed phenotypes, muscle strength, disease severity/progression, and onset ages at different endpoints (mainly obtained from patients' records or recollections). Phenotypes of FSHD1 were classified by the Comprehensive Clinical Evaluation Form (CCEF); patients who were classified as category C were considered asymptomatic/unaffected patients.[13] Muscle strength was assessed by FSHD clinical score (CS). Patients with severe facial involvement were diagnosed as a CS score of 2 on the facial muscles section; and patients with severe upper extremity (UE) involvement were diagnosed as a CS score of 3 on the scapular girdle muscles section or a score of 2 on the

upper limb muscles section.[14] Disease severity/progression was assessed by implementing specific scales: 1) FSHD CSS; 2) age-corrected CSS (ACSS), adjusted for the patient's age at examination according to the following formula: ((CSS×2)/age at examination) × 1000;[15, 16] and 3) mRS, a simple 6-point assessment that included a reference to limitation in activity.[17]

Statistical analysis

For baseline characteristics, continuous parametric variables were expressed as mean (SDs), whereas continuous nonparametric variables were expressed as median (range). Categorical data were given as a percentage (%). Whether variables were normally distributed or not was confirmed by Kolmogorov-Smirnov tests or Shapiro-Wilk's test. Then, nonparametric continuous variables were compared with the Mann-Whitney U test (for 2 groups) or Kruskal-Wallis H test (for > 2 groups; statistical significance value was adjusted by Bonferroni correction); an independent t-test was used for continuous variables with a normal distribution. A χ^2 test (Fisher's exact test when the expected value was < 5) was used to compare categorical variables. Correlations between variables were assessed with the Spearman rank correlation coefficient.

The area under the curve (AUC) of receiver operating characteristic (ROC) curves was used to assess the ability of distal D4Z4 hypomethylation within the 4qA-specific allele to predict diagnosis and disease severity/progression between FSHD1 patients and healthy controls as well as between symptomatic FSHD1 patients and asymptomatic/unaffected FSHD1 patients.

Kaplan-Meier curves were used to compare the cumulative probability risk of motor function progression of lower extremity involvement and independent ambulation loss during the follow-up period between patients with genetically confirmed FSHD1 with different degrees of distal D4Z4 hypomethylation. A log-rank test was used to determine any statistical differences in the Kaplan-Meier curves between the groups.

Cox proportional hazards regression models were used initially to obtain hazard ratios (HRs) and 95% confidence intervals (CIs) in the three groups with relatively low methylation (LM1, LM2, and LM3) compared to the group with relatively high methylation (HM) at both outcomes of lower extremity involvement and independent ambulation loss. Then multivariable-adjusted models (adjusted for age at examination [< 20 years old] [continuous variable], sex [categorical data], D4Z4 RU [continuous variable],

and haplotypes of 4qA/A and 4qA/B [categorical data]) were performed to assess the association between distal D4Z4 hypomethylation and disease progression.

Statistical analyses were performed in SPSS version 22 (IBM SPSS Inc, Chicago IL). The level of statistical significance was set at $p \leq 0.05$.

Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

Baseline characteristics

Overall, 1164 total participants were included in the cohort, 823 of whom were FSHD1 patients, genetically confirmed by PFGE-based Southern blotting, and 341 were healthy controls. Age at examination varied widely among the 823 FSHD1 patients, ranging from 2 to 81 years (median 33); 383 (46.5%) were women. Median length of the contracted D4Z4 repeat region was 23.5 kb (range 10.0–37.0 kb), and contained 5 units (range 1–9) after formula conversion. The distribution of distal D4Z4 methylation levels was highly variable, ranging from 2.7% to 89.6%. Clinically, 722 (87.7%) patients were symptomatic (category A, B, and D) and 101 (12.3%) were asymptomatic (category C) according to CCEF; clinical scores ranged from normal to severe, with CS ranging from 0 to 15 points (median 6), CSS ranging from 0 to 5 points (median 3), and ACSS ranging from 0 to 1500 points (median 153.8). Of the 722 symptomatic FSHD1 patients, onset age of first-ever muscle weakness varied widely, from 1 to 81 years (median 16) (Table 1).

Diagnostic prediction ability of distal D4Z4 hypomethylation

To verify whether distal D4Z4 DNA methylation levels were predictive of FSHD1 status, we compared distal D4Z4 methylation levels between FSHD1 patients and healthy controls. Notably, the methylation levels of all 10 CpGs, as well as the average of the 10 CpGs, were significantly lower in FSHD1 patients

than in healthy controls (all $p < 0.0001$; Figure 1A). ROC analysis revealed that the methylation levels of all 10 CpGs had a relatively large AUC, ranging from 0.8201 to 0.9684. In particular, CpG6 methylation levels were the most informative, showing the largest AUC of 0.9684 (0.9584–0.9785, $p < 0.0001$); at a cut-off value of 72.79%, it provided a sensitivity of 92.4% and specificity of 90.8% for distinguishing between FSHD1 patients and healthy controls (Figure 1B). Further analysis among the 823 FSHD1 patients showed that the methylation levels of all 10 CpGs (and their average value) were significantly lower in symptomatic patients compared to that in asymptomatic/unaffected patients (all $p < 0.0001$; Figure 1A). In addition, the methylation level of CpG6 showed the largest AUC of 0.7417 (0.6903–0.7931, $p < 0.0001$), and at a cut-off value of 67.48%, sensitivity and specificity for distinguishing between symptomatic and asymptomatic/unaffected FSHD1 patients were relatively modest at 66.3% and 74.7%, respectively (Figure 1C). The detailed data are provided in eTable 1.

Prediction of lower extremity involvement with distal D4Z4 hypomethylation

Among the 823 FSHD1 patients, 324 (39.4%) had lower extremity involvement while 499 (60.6%) showed no such involvement. FSHD1 patients with involvement of lower extremities had significantly lower CpG6 methylation levels (median 54.20%; range 14.35%–76.66%) than patients with severe facial or upper extremity involvement (median 63.71%; range 20.39%–81.95%); while among the patients with no lower extremity involvement, there was no difference in CpG6 methylation levels between patients with severe facial or upper extremity involvement and patients without severe facial or upper extremity involvement ($p = 0.999$, Kruskal-Wallis H test; Figure 2A). We then examined whether CpG6 methylation levels could predict FSHD1 disease progression to lower extremity involvement. At a CpG6 methylation cut-off of 65.97%, ROC analysis showed a modest sensitivity of 43.9% and a specificity of 77.0% for distinguishing between FSHD1 with or without involvement of lower extremities, with an AUC of 0.6386 (0.5816–0.6956, $p < 0.0001$) (Figure 2B).

Association between distal D4Z4 methylation level and disease severity

In evaluating FSHD1 disease severity, we observed that both CS and ACSS decreased as CpG6 methylation levels increased. Specifically, median CS values were 7 (range 0–14), 7 (range 0–15), 5 (range 0–14), and 2

(range 0–13) for the LM1, LM2, LM3, and HM groups, respectively ($p < 0.0001$, Kruskal-Wallis H test); median ACSS values were 218.8 (range 0.0–1500), 166.7 (range 0.0–444.4), 142.9 (range 0.0–700.0), and 71.4 (range 0.0–875.0) for the LM1, LM2, LM3, and HM groups, respectively ($p < 0.0001$, Kruskal-Wallis H test) (Table 2). In general, CpG6 methylation levels were negatively correlated with both CS (Spearman's r = -0.392, $p < 0.0001$) and ACSS (Spearman's r = -0.432, $p < 0.0001$) (Figure 3, A–B). Furthermore, categorizing each FSHD1 patient as either the 4qA/A or 4qA/B haplotype, we found that CpG6 methylation was negatively correlated with ACSS in both groups (4qA/A haplotype: Spearman's r = -0.461, $p < 0.0001$; 4qA/B haplotype: Spearman's r = -0.479, $p < 0.0001$) (eFigure 1, A–B; eTable 2).

Since onset age of first-ever muscle weakness is an indicator of FSHD1 disease severity, we next investigated whether it was correlated with varying degrees of CpG6 methylation. In general, CpG6 methylation levels were positively correlated with onset age of first-ever muscle weakness (Spearman's r = 0.297, $p < 0.0001$) (Figure 3C), with median onset ages of first-ever muscle weakness of 15 (range 2–50), 16 (range 1–52), 16 (range 1–63), and 21 (range 5–81) for the LM1, LM2, LM3, and HM groups, respectively ($p < 0.0001$, Kruskal-Wallis H test) (Table 2). A weak correlation between CpG6 methylation levels and age at examination was detected in the healthy controls (Spearman's r = 0.067, $p = 0.3439$) (eFigure 1C)

In addition, CpG6 methylation levels were also positively correlated with D4Z4 RU number (Spearman's r = 0.257, $p < 0.0001$). In order to analyze possible associations between CpG6 methylation and clinical parameters, we first divided FSHD1 patients according to D4Z4 RU number, from 1 to 9. Among FSHD1 patients carrying fewer (1–3) D4Z4 RUs (n = 231), CpG6 hypomethylation was mild or modest and significantly correlated with onset age of first-ever muscle weakness (Spearman's r = 0.371, $p < 0.001$), CS (Spearman's r = -0.347, $p < 0.001$), and ACSS (Spearman's r = -0.503, $p < 0.001$). Similarly, CpG6 methylation level was also significantly correlated with onset age of first-ever muscle weakness, CS, ACSS in FSHD1 patients carrying an intermediate (4–6) or large (7–9) D4Z4 RU number (eTable 3).

Association between distal D4Z4 hypomethylation and disease progression

Clinical assessments identified 324 (44.9%) of 823 FSHD1 patients with lower extremity involvement (CSS ≥ 3.0). The proportion of patients with lower extremity involvement decreased as CpG6 methylation levels increased in patients, while onset age of lower extremity involvement increased along with methylation levels. Specifically, 52.9%, 44.2%, 36.9%, and 23.4% of patients in the LM1, LM2, LM3, and HM groups, respectively, were patients with lower extremity involvement ($p < 0.0001$, Kruskal-Wallis H test); onset ages

of lower extremity involvement in these groups were 20 (range 5–65), 26.5 (range 5–54), 25 (range 8–59), and 26.5 (range 12–60), respectively ($p < 0.0001$; Kruskal-Wallis H test) (Table 2). Methylation level of CpG6 was also generally positively correlated with onset age of lower extremity involvement (Spearman's r = 0.247, $p < 0.0001$; Figure 3D).

Log-rank (Mantel-Cox) tests of these patients with lower extremity involvement showed a median age at examination of 31 years for LM1 patients (95% CI 26–36), 39 years for LM2 (95% CI 36–42), 42 years for LM3 (95% CI 36–48), and 61 years for HM patients (95% CI 58–64). In addition, loss of independent ambulation occurred at a median age of 31 years (95% CI 23–39) in group LM1, 29 years in group LM2 (95% CI 21–37), 35 years in group LM3 (95% CI 27–43), and 52 years in HM patients (95% CI 47–58) (Figure 4). Similar trends were evident in Kaplan-Meier curves for FSHD1 patients carrying 1–3 D4Z4 RUs, 4–6 D4Z4 RUs, and 7–9 D4Z4 RUs (eFigure 2).

Compared with the 205 HM group patients, the 618 patients with relatively lower CpG6 methylation levels in groups LM1, LM2, and LM3 had generally increased risk of progressing to lower extremity involvement in Cox regression analyses ($p < 0.0001$), corresponding to HRs of 3.811 (95%CI 2.703–5.373, $p < 0.0001$), 2.659 (95%CI 1.891–3.841, $p < 0.0001$), and 2.109 (95%CI 1.464–3.037, $p < 0.0001$), respectively. After adjusting for sex, age at examination, contracted D4Z4 RU number, and 4qA/A or 4qA/B haplotype classification, the LM1, LM2, and LM3 groups still showed a significantly higher risk of progressing to lower extremity involvement, with adjusted HRs of 3.510 (95% CI 2.289–5.382, $p < 0.0001$), 2.664 (95% CI 1.735–4.092), and 2.017 (95% CI 1.324–3.075, $p = 0.001$), respectively. Furthermore, the results of Cox regression analyses were consistent with lower CpG6 methylation associated with higher HRs for progressing to independent ambulation loss. Compared with the HM group, the LM1, LM2, and LM3 groups had adjusted HRs of 3.523 (95% CI 1.565–7.930, $p = 0.002$), 3.356 (95% CI 1.458–7.727, $p = 0.004$), and 2.956 (95% CI 1.245–7.020, $p = 0.017$), respectively (Table 3).

To verify whether lower methylation levels promote a rapid disease progression, we followed up 30 patients in each of the LM1 and HM groups. After a median follow-up period of 5 years (range 3–9), patients in the LM1 group displayed higher CS increases of (3 points; range 1–4) and higher age-corrected CSS increases (34.2 points; range -46.6–176.5) than patients in the HM group (CS: 1 point; range 0–22, age-corrected CSS: 4.8 points; range -54.9–105.3) (all $p < 0.001$) (eFigure 3). 8 of 20 patients in the LM1 group developed lower extremity involvement, of which 1 patient progressed to independent ambulation loss, while only 2 of the 23 patients in the HM group developed lower extremity involvement. eTable 4 shows

detailed clinical data for both groups at baseline and follow-up.

Discussion

Findings in this study support the conclusions of previous studies that suggest the degree of hypomethylation at distal D4Z4 RU is a determinant of disease penetrance. In addition, the current study also demonstrates that the degree of distal D4Z4 hypomethylation reflects disease severity and progression to involvement of lower extremity motor function in a large cohort of patients with genetically confirmed FSHD1. FSHD1 patients with lower methylation levels in distal D4Z4 microsatellites display more severe muscle weakness and a higher incidence of lower extremity involvement at a younger age compared to those with relatively higher methylation levels. Furthermore, in a follow-up of these FSHD1 patients, we found that lower distal D4Z4 methylation levels could also predict a higher risk of progression to lower extremity involvement and independent ambulation loss. Together, these findings suggest that measurement of distal D4Z4 methylation levels may be informative for the clinical identification of patients at risk for early lower extremity involvement and progression.

Other studies examining the influence of genetic factors on disease phenotype have previously established that contracted 4qA D4Z4 RU number is inversely correlated with the severity of clinical manifestations of FSHD1.[15, 18] However, it is notable that FSHD1 has a characteristically high variability in disease progression, such as incomplete penetrance and a marked interindividual and intrafamilial heterogeneity.[19, 20] Thus, contracted D4Z4 RU number alone is insufficient to predict the severity or progression of clinical manifestations at all times. To address this issue, we examined differences in 4qA-allele-specific hypomethylation and found that it can accurately predict disease severity and progression in genetically confirmed FSHD1 patients.

BSS analysis provides an averaged representation of a methylation state at each cytosine residue and has become the gold-standard method for studying CpG methylation.[9,10] In this study, BSS was used to assess methylation levels at a single base resolution across the most distal D4Z4 repeat region in patients with the disease-permissive 4qA haplotype in order to identify links between FSHD1 diagnosis/prognosis and epigenetic data of distal D4Z4 methylation levels. Consistent with previous reports,[11] this assay revealed that CpG6 methylation level, in particular, was the most informative, showing the greatest difference between 823 FSHD1 samples and 341 healthy controls, as well as between 722 symptomatic and 101 asymptomatic/unaffected FSHD1 patients, with AUCs of 0.9684 and 0.7417, respectively, in ROC curve analysis. Our findings also confirmed that reduced DNA methylation of D4Z4 RU is a reliable marker for

FSHD diagnosis, as proposed in previous studies.[11] However, there are several control subjects that showed lower mean CpG methylation levels compared to FSHD1 patients. Methylation detection errors could be relevant here. Additionally, it merits consideration that FSHD1 patients carrying the 4qA/4qA haplotype could have their methylation levels overestimated, owing to methylation of both 4qA alleles. Similar issues with inconclusive diagnostic analysis of FSHD based on detection of 4qA methylation levels have also been noted in recent studies by Erdmann et al. and Caputo et al.[21,25]

One recent study proposed that D4Z4 hypomethylation could serve as a potentially important prognostic marker for FSHD,[21] but contradicted another report that found no correlation between D4Z4 methylation levels assessed by MSRE and clinical manifestation or disease severity.[22] In the present study, we stratified patients according to CpG6 methylation for univariate analysis of disease severity, which indicated that muscle weakness (measured by CS and ACSS) is more severe and onset age of first-ever muscle weakness is earlier in patient groups with lower methylation levels (groups of LM1, LM2, and LM3). Specifically, LM1 group patients, who had the highest degree of hypomethylation, also showed the most severe muscle weakness and the earliest onset age at first-ever muscle weakness. Additionally, our correlation analysis indicated that the degree of distal D4Z4 hypomethylation is positively associated with CS and ACSS scores, and negatively associated with onset age of first-ever muscle weakness. These associations remained significant in univariate correlation analysis that also accounted for the 4qA/A and 4qA/B haplotypes. No apparently informative correlation was detected between CpG6 methylation levels and age at examination in the healthy controls, indicating no significant age-effect on methylation level changes. These above observations are consistent with another recent study that demonstrated distal D4Z4 methylation level can be an indicator of disease severity, in addition to its ability to differentiate FSHD1 from healthy controls.[21]

Among all FSHD1 patients carrying 1 to 9 D4Z4 RU in this cohort, general analysis could not determine whether differences in distal D4Z4 methylation could influenced disease severity independent of D4Z4 RU number. Thus, further analysis of correlations between distal D4Z4 methylation levels and CS, ACSS, and onset age of first-ever muscle weakness in patients with the same contracted D4Z4 RU number showed that distal D4Z4 methylation levels were negatively correlated with CS and ACSS, but positively correlated with onset age of first-ever muscle weakness for all FSHD1 patients with 1–3, 4–6, or 7–9 D4Z4 RUs. These findings indicated that epigenetic regulation may indeed independently influence disease severity in FSHD1.

Lower extremity involvement is well-understood to be a pre-requisite leading to independent ambulation

loss and eventual progression to wheelchair dependency in FSHD1.[23, 24] Although our previous study suggested that differences in distal D4Z4 hypomethylation did not appear to affect the rate of progression to wheelchair dependence (*i.e.,* advanced impairment of motor function) (*p* = 0.07),[4] our current understanding is now expanded by evidence showing that differences in distal D4Z4 hypomethylation may reflect progression to lower extremity involvement and loss of independent ambulation in early stages of impaired motor function in FSHD1. We found that FSHD1 patients with lower distal D4Z4 methylation levels had a higher incidence of progression to lower extremity involvement and at a younger age than FSHD1 patients with relatively higher methylation levels (Table 2 and Figure 4A). These patients with lower distal D4Z4 methylation levels were also likely to lose independent ambulation at a younger age (Figure 4B), especially those in the LM1 group, which had the greatest extent of hypomethylation and the highest proportion and earliest onset age of lower extremity involvement. Consistent with this finding, the LM1 group contained the highest proportion of disability/wheelchair dependence (*p* < 0.001). Further multivariate Cox regression model showed that greater hypomethylation at distal D4Z4 was associated with higher HRs for motor symptom progression to lower extremity involvement and independent ambulation loss (adjusted for age at examination, sex, D4Z4 RU, and 4qA/A and 4qA/B haplotypes). In addition, given the chronic disease progression of FSHD[26], we performed a follow-up (median 5 years) between the LM1 and HM groups (*i.e.*, those groups with the greatest difference of methylation levels). Interestingly, the follow-up data indicated that the LM1 group had more pronounced increases in disease severity and a higher proportion of lower extremity involvement than the HM group, indicating that lower methylation levels do promote more rapid disease progression. These results suggest that a higher extent of distal D4Z4 hypomethylation may increase the rate of motor function progression to lower extremities.

This study has several limitations. Most importantly, assessment of correlations between distal D4Z4 methylation levels and disease severity and progression cannot be compared with other studies examining D4Z4 methylation due to the selection of different regions within the D4Z4 repeat region. However, our analysis captured the most distal D4Z4 repeat and the adjacent A-type locus, which has been correlated with pathological expression of *DUX4* in contracted D4Z4 regions. This region was also examined by Calandra et al.[11] Another limitation is that the final distal methylation levels might be diluted in FSHD1 patients with the 4qA/A haplotype due to the inclusion of non-contracted D4Z4 repeats. However, no significant differences were identified between the results of correlation analysis for the 4qA/A haplotype and those of the 4qA/B haplotype. Finally, distal D4Z4 methylation levels were checked only when the participants were enrolled in the study. Future studies will include serial follow-ups to quantify changes in distal D4Z4

methylation levels along with evaluations of motor function to further delineate changes in D4Z4 hypomethylation that occur over the course of FSHD1.

In summary, our results suggest that distal D4Z4 methylation levels detected by BSS could can serve as a noninvasive, easily accessible biomarker to predict disease severity, as well as a potentially prognostic marker for FSHD1. Consistent with the conclusions of previous studies, our data support that D4Z4 hypomethylation is a reliable auxiliary diagnostic indicator for use in FSHD diagnostic workflows. Future prospective follow-up study designs that incorporate other factors such as contracted D4Z4 repeat units and/or disease duration should help to develop a prognostic risk model for FSHD and provide evidence to guide the management of patients with different methylation levels.

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WNL-2023-000256_etab4 ---http://links.lww.com/WNL/C847

References

1. Wang LH, Tawil R. Facioscapulohumeral Dystrophy. *Curr Neurol Neurosci Rep.* 2016;16(7):66; doi:10.1007/s11910-016-0667-0

2. Tawil R, Van Der Maarel SM. Facioscapulohumeral muscular dystrophy. *Muscle Nerve.* 2006;34(1):1-15; doi:10.1002/mus.20522

3. Wang Z, Qiu L, Lin M, et al. Prevalence and disease progression of genetically-confirmed facioscapulohumeral muscular dystrophy type 1 (FSHD1) in China between 2001 and 2020: a nationwide population-based study. *Lancet Reg Health West Pac.* 2021;18:100323; doi:10.1016/j.lanwpc.2021.100323

4. Qiu L, Chen L, Zheng F, et al. Wheelchair use in genetically confirmed FSHD1 from a large cohort study in Chinese population. *Brain.* 2022;145(6):e51-e54; doi:10.1093/brain/awac097

5. Katz NK, Hogan J, Delbango R, Cernik C, Tawil R, Statland JM. Predictors of functional outcomes in patients with facioscapulohumeral muscular dystrophy. *Brain.* 2021;144(11):3451-3460; doi:10.1093/brain/awab326

6. Himeda CL, Jones PL. The Genetics and Epigenetics of Facioscapulohumeral Muscular Dystrophy. *Annu Rev Genomics Hum Genet.* 2019;20:265-291; doi:10.1146/annurev-genom-083118-014933

7. Hewitt JE, Lyle R, Clark LN, et al. Analysis of the tandem repeat locus D4Z4 associated with facioscapulohumeral muscular dystrophy. *Hum Mol Genet.* 1994;3(8):1287-1295; doi:10.1093/hmg/3.8.1287

8. de Greef JC, Wohlgemuth M, Chan OA, et al. Hypomethylation is restricted to the D4Z4 repeat array in phenotypic FSHD. *Neurology.* 2007;69(10):1018-1026; doi:10.1212/01.wnl.0000271391.44352.fe

9. Salsi V, Magdinier F, Tupler R. Does DNA Methylation Matter in FSHD?. *Genes (Basel).* 2020;11(3):258; doi:10.3390/genes11030258

10. Jones TI, King OD, Himeda CL, et al. Individual epigenetic status of the pathogenic D4Z4 macrosatellite correlates with disease in facioscapulohumeral muscular dystrophy. *Clin Epigenetics.* 2015;7(1):37; doi:10.1186/s13148-015-0072-6

11. Calandra P, Cascino I, Lemmers RJ, et al. Allele-specific DNA hypomethylation characterises FSHD1 and FSHD2. *J Med Genet.* 2016;53(5):348-355; doi:10.1136/jmedgenet-2015-103436

12. Qiu L, Ye Z, Lin L, et al. Clinical and genetic features of somatic mosaicism in facioscapulohumeral dystrophy. *J Med Genet.* 2020;57(11):777-785; doi:10.1136/jmedgenet-2019-106638

13. Ricci G, Ruggiero L, Vercelli L, et al. A novel clinical tool to classify facioscapulohumeral muscular dystrophy phenotypes. *J Neurol.* 2016;263(6):1204-1214; doi:10.1007/s00415-016-8123-2

14. Lamperti C, Fabbri G, Vercelli L, et al. A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: The FSHD clinical score. *Muscle Nerve*. 2010;42(2):213-217; doi:10.1002/mus.21671

15. Ricci E, Galluzzi G, Deidda G, et al. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype. *Ann Neurol.* 1999;45(6):751-757; doi:10.1002/1531-8249(199906)45:6<751::aid-ana9>3.0.co;2-m

16. van Overveld PG, Enthoven L, Ricci E, et al. Variable hypomethylation of D4Z4 in facioscapulohumeral muscular dystrophy. *Ann Neurol*. 2005;58(4):569-576; doi:10.1002/ana.20625

17. Wilson JT, Hareendran A, Grant M, et al. Improving the assessment of outcomes in stroke: use of a structured interview to assign grades on the modified Rankin Scale. *Stroke*. 2002;33(9):2243-2246; doi:10.1161/01.str.0000027437.22450.bd

18. Zernov N, Skoblov M. Genotype-phenotype correlations in FSHD. *BMC Med Genomics*. 2019;12(Suppl 2):43; doi:10.1186/s12920-019-0488-5

19. Tonini MM, Passos-Bueno MR, Cerqueira A, Matioli SR, Pavanello R, Zatz M. Asymptomatic carriers and gender differences in facioscapulohumeral muscular dystrophy (FSHD). *Neuromuscul Disord*. 2004;14(1):33-38; doi:10.1016/j.nmd.2003.07.001

20. Pastorello E, Cao M, Trevisan CP. Atypical onset in a series of 122 cases with FacioScapuloHumeral Muscular Dystrophy. *Clin Neurol Neurosurg*. 2012;114(3):230-234; doi:10.1016/j.clineuro.2011.10.022

21. Erdmann H, Scharf F, Gehling S, et al. Methylation of the 4q35 D4Z4 repeat defines disease status in facioscapulohumeral muscular dystrophy. *Brain*. 2022;awac336; doi:10.1093/brain/awac336

22. Nikolic A, Jones TI, Govi M, et al. Interpretation of the Epigenetic Signature of Facioscapulohumeral Muscular Dystrophy in Light of Genotype-Phenotype Studies. *Int J Mol Sci*. 2020;21(7):2635; doi:10.3390/ijms21072635

23. Vercelli L, Mele F, Ruggiero L, et al. A 5-year clinical follow-up study from the Italian National Registry for FSHD. *J Neurol*. 2021;268(1):356-366; doi:10.1007/s00415-020-10144-7

24. Monforte M, Laschena F, Ottaviani P, et al. Tracking muscle wasting and disease activity in facioscapulohumeral muscular dystrophy by qualitative longitudinal imaging. *J Cachexia Sarcopenia Muscle*. 2019;10(6):1258-1265; doi:10.1002/jcsm.12473

25. Caputo V, Megalizzi D, Fabrizio C, et al. D4Z4 Methylation Levels Combined with a Machine Learning Pipeline Highlight Single CpG Sites as Discriminating Biomarkers for FSHD Patients. *Cells*. 2022;11(24):4114; doi:10.3390/cells11244114

26. Tihaya MS, Mul K, Balog J, et al. Facioscapulohumeral muscular dystrophy: the road to targeted therapies. *Nat Rev Neurol*. 2023;19(2):91-108; doi:10.1038/s41582-022-00762-2

Tables

Table 1. Baseline characteristics of FSHD1 participants

Characteristics	Total (n = 823)
Demographic	
Sex, female, n (%)	383 (46.5)
Age at examination, y, median (range)	33 (2-81)
Genetic/epigenetic	
Length of contracted D4Z4 repeats array, kB, median (range)	23.5 (10.0-37.0)
Number of contracted D4Z4 repeat units, median (range)	5 (1-9)
Distal D4Z4 methylation level, percentage, median (range)[a]	
CpG1 locus	23.1 (2.7-63.2)
CpG2 locus	55.5 (22.3-87.2)
CpG3 locus	19.1 (5.2-52.1)
CpG4 locus	38.0 (9.5-76.8)
CpG5 locus	32.3 (6.6-69.0)
CpG6 locus	60.4 (13.7-95.0)
CpG7 locus	55.5 (17.3-89.6)
CpG8 locus	46.2 (8.8-87.3)
CpG9 locus	38 (11.4-76.4)
CpG10 locus	38.6 (11.1-76.9)
Average of the 10 CpGs locus	40.8 (13.8-69.4)
Clinical	
Phenotypic classification (based on CCEF), n (%)	
Category A, B, and D (symptomatic)	722 (87.7)
Onset age of first-ever muscle weakness, y, median (range)	16 (1-81)
Category C (asymptomatic/unaffected)	101 (12.3)
Assessments, median (range)	
FSHD clinical score (CS, 0–15) (evaluated participants = 471)	6 (0-15)
Clinical severity scale (CSS, 0–5) (evaluated participants = 431)	3 (0-5)
Age-corrected CSS (ACSS, 0–10,000) (evaluated participants = 431)	153.8 (0-1500)
Motor function	
Lower extremity involvement, n/the symptomatic patients (%)	324/722 (44.9)
Onset age of lower extremity involvement, y, median (range)	24 (5-65)
Wheelchair dependency, n/the patients of lower extremity involvement (%)	55/324 (17.0)

[a] Representing the regional methylation status of the most distal D4Z4 repeat units on the permissive haplotypes 4qA.

Abbreviation: CCEF = the Comprehensive Clinical Evaluation Form; FSHD1 = Facioscapulohumeral muscular dystrophy type 1.

Table 2. Baseline characteristics of FSHD1 groups with different degree of distal D4Z4 methylation

Characteristics	LM1 group (n = 206)	LM2 group (n = 206)	LM3 group (n = 206)	HM group (n = 205)	p Value[a]
Demographic					
Sex, female, n (%)	112 (54.4)	85 (41.3)	87 (42.2)	99 (48.3)	**0.027**
Age at examination, y, median (range)	28 (3-67) [***]	32 (2-77) [***]	31 (2-72) [***]	42 (4-81)	**<0.0001**

Genetic					
Number of contracted D4Z4 repeat units, median (range)	4 (1-9) [***]	5 (2-9)	4 (2-8)	5 (2-9)	**<0.0001**
Clinical					
Phenotypic classification (based on CCEF), n (%)					
Category A, B, and D (symptomatic)	198 (96.1)	192 (93.2)	182 (88.3)	69 (73.2)	
Onset age at first-ever muscle weakness, y, median (range)	15 (2-50) [***]	16 (1-52) [***]	16 (1-63) [***]	21 (5-81)	**<0.0001**
Category C (asymptomatic/unaffected)	8 (3.9) [***]	14 (6.8) [***]	24 (11.7) [***]	55 (26.8) [***]	**<0.0001**
Assessments, median (range)	Evaluated participants = 127	Evaluated participants = 131	Evaluated participants = 132	Evaluated participants = 137	
FSHD clinical score (CS, 0–15)	7 (0-14) [***]	7 (0-15) [***]	5 (0-14) [***]	2 (0-13)	**<0.0001**
Clinical severity scale (CSS, 0–5)	3.5 (0.0-4.5) [***]	3.0 (0.0-5.0) [***]	3.0 (0.0-5.0) [**]	1.5 (0.0-5.0)	**<0.0001**
Age-corrected CSS (ACSS, 0–10,000)	218.8 (0.0-1500) [***]	166.7 (0.0-444.4) [***]	142.9 (0.0-700.0) [***]	71.4 (0.0-875.0)	**<0.0001**
Motor function					
Lower extremity involvement, n (%)	109 (52.9) [***]	91 (44.2) [*]	76 (36.9)	48 (22.4)	**<0.0001**
Onset age of lower extremity involvement, y, median (range)	20 (5-65) [**]	26.5 (5-54)	25 (8-59)	26.5 (12-60)	**<0.0001**
Wheelchair dependency, n (%)	21 (16.5) [***]	20 (14.6) [***]	11 (8.3) [***]	3 (2.2)	**<0.0001**
Onset age of wheelchair dependency, y, median (range)	32 (9-64)	30 (16-67)	36 (25-55)	52 (20-82)	0.209

[a]p values were based on Kruskal-Wallis H test for continuous variables and χ^2 test (or Fisher's exact test) for frequencies.

[*]p value <0.05; [**]p value <0.001; [***]p value <0.0001. Values in bold indicate p value < 0.05.

Abbreviation: LM1 group = the group 1 with the low methylation, first quartile; LM2 group = the group 2 with the low to intermediate hypomethylation, second quartile; LM3 group = the group 3 with the intermediate to high methylation, third quartile; HM group = the group 4 with the high methylation, fourth quartile.

Table 3. Risk analysis of disease progression for FSHD1 with different distal D4Z4 hypomethylation levels

	LM1 group (n = 206)		LM2 group (n = 206)		LM3 group (n = 206)		p value
Unadjusted hazard ratios, HR (95% CI) for outcomes, p value							
Lower extremity involvement[a]	3.811 (2.703-5.373)	**<0.0001**	2.695 (1.891-3.841)	**<0.0001**	2.109 (1.464-3.037)	**<0.0001**	**<0.0001**
Independent ambulation loss[b]	2.263 (1.169-4.379)	**0.015**	2.327 (1.157-4.682)	**0.018**	1.994 (0.994-4.212)	0.070	0.075
Adjusted hazard ratios, aHR (95% CI) for outcomes, p value							
Lower extremity involvement[a]	3.510 (2.289-5.382)	**<0.0001**	2.664 (1.735-4.092)	**<0.0001**	2.017 (1.324-3.075)	**0.001**	**<0.0001**
Independent ambulation loss[b]	3.523 (1.565-7.930)	**0.002**	3.356 (1.458-7.727)	**0.004**	2.956 (1.245-7.020)	**0.014**	**0.017**

[a]Evaluated objectively and professionally with the CSS ≥ 3 at study initiation.
CSS ≥ 3.0 cases for n = 324: LM1 group (n = 109); LM2 group (n = 91); LM3 group (n = 76) and HM group (n = 48).

[b]Evaluated objectively and professionally with the mRS ≥ 4 and the CSS≥ 4.5 during follow-up.
mRS ≥ 4 and the CSS≥ 4.5 cases for n = 84: LM1 group (n = 30); LM2 group (n = 23); LM3 group (n = 17) and HM group (n = 14).

Values in bold indicate p value < 0.05.

Abbreviation: CI = confidence intercal; CSS = clinical severity score; mRS = the modified Rankin Scale. LM1 group = the group 1 with the low methylation, first quartile; LM2 group = the group 2 with the low to intermediate hypomethylation, second quartile; LM3 group = the group 3 with the intermediate to high methylation, third quartile; HM group = the group 4 with the high methylation, fourth quartile.

Figure legends

Figure 1 Diagnostic accuracy and penetrance prediction of distal D4Z4 hypomethylation status. (A) Percentage of distal D4Z4 methylation levels of 10 CpGs analyzed in healthy controls (n = 341, green bar), symptomatic FSHD1 patients (n = 722, purple bar), and asymptomatic/unaffected FSHD1 patients (n = 101, light yellow bar). Bars present the mean value ± SD; the "average" here reports the average value of all 10 CpGs assessed. Methylation levels were compared by the Kruskal-Wallis *H* test with Bonferroni correction. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$. (B, C) ROC curve analyses for differentiating between (B) all FSHD1 patients (n = 823, black curve) and healthy controls (green curve) and (C) symptomatic FSHD1 patients (purple curve) and asymptomatic/unaffected FSHD1 patients (light yellow curve) according to the CpG6 methylation level. FSHD1, facioscapulohumeral dystrophy type 1; ROC, receiver operating characteristic; SD, standard deviation.
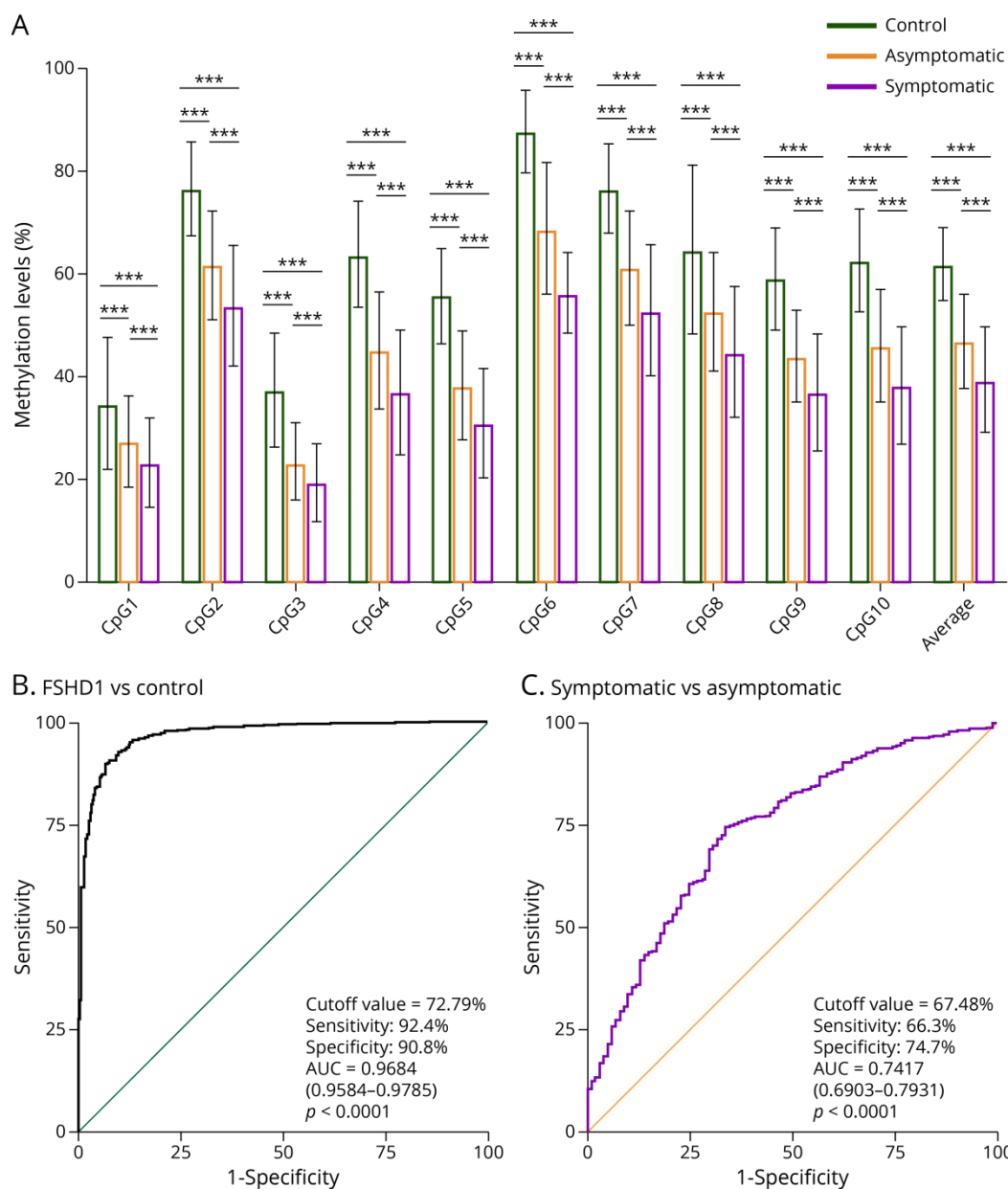
Figure 2 Lower extremity involvement prediction ability of distal D4Z4 hypomethylation. (A) Methylation level of CpG6 for FSHD1 patients with no involvement of lower extremity (LE) (total n = 139, patients had detail FSHD clinical scores) and FSHD1 patients with involvement of LE but that can walk independently (n = 252, purple circles). FSHD1 patients with no LE involvement were further divided into two subgroups: patients with no severe involvement of face or upper extremity (n = 96, green circles) and patients with severe involvement of face or upper extremity (n = 43, blue circles). Bars present the median (range). Methylation levels of CpG6 were compared using a Kruskal-Wallis *H* test with Bonferroni correction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. (B) ROC curve analyses for differentiating between symptomatic FSHD1 patients with involvement of LE (purple curve) and symptomatic/unaffected FSHD1 patients with no involvement of LE (blue curve). FSHD1, facioscapulohumeral dystrophy type 1;

LE: lower extremity; ROC, receiver operating characteristic.
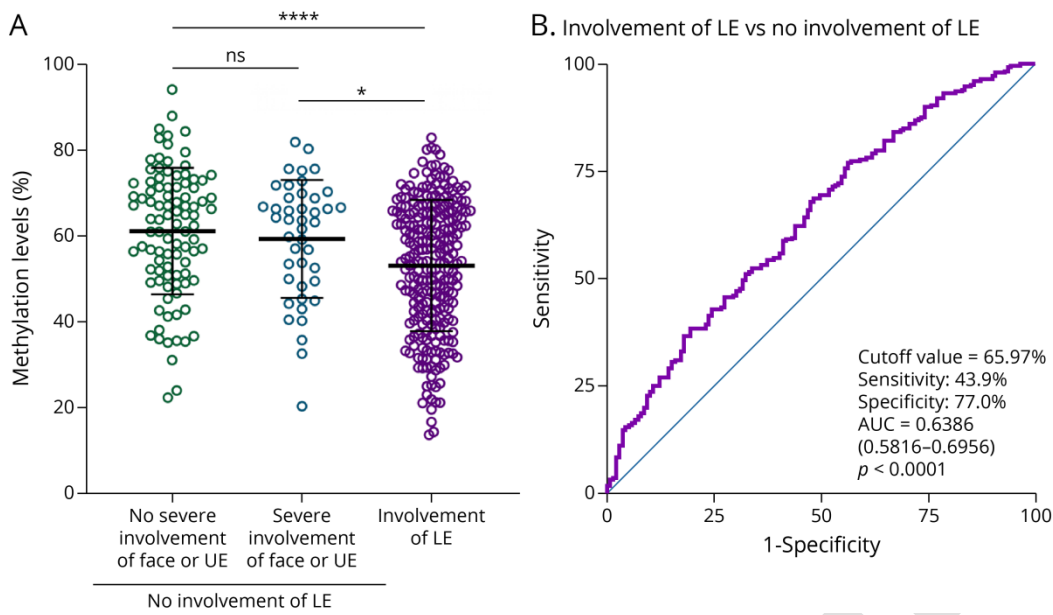

Figure 3 Correlations between methylation levels of CpG6 and clinical parameters. (A, B) Correlations between methylation levels of CpG6 and (A) FSHD clinical score and (B) age-corrected CSS in all patients with methylation assessed. (C, D) Correlations between methylation levels of CpG6 and (C) onset age of first-ever muscle weakness and (D) onset age of lower extremity involvement in all patients with methylation assessed.
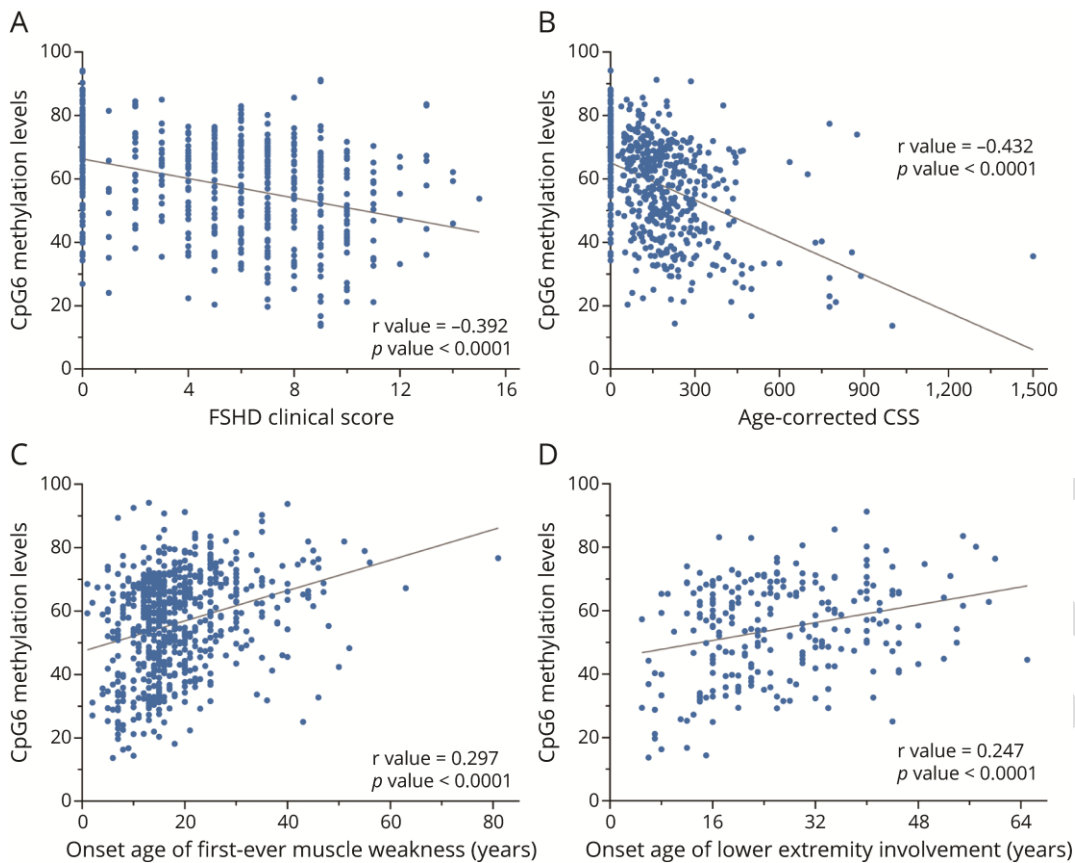
Figure 4 Kaplan-Meier curves showing outcomes for lower extremity involvement and independent ambulation loss in FSHD1 patients with different degrees of D4Z4 methylation levels. (A) Significant differences in outcomes for lower extremity involvement were analyzed by log-rank (Mantel-Cox) tests among FSHD1 patients stratified into four groups depending on methylation percentage quartiles: group 1 with low methylation (LM1), group 2 with low to intermediate methylation (LM2), group 3 with intermediate to high methylation (LM3), and group 4 with high methylation (HM). (B) Significant differences in outcomes for independent ambulation loss among the four groups were analyzed by log-rank (Mantel-Cox) tests (all $p < 0.0001$).

A

Probability of lower extremity involvement (%)

Age at examination (years)
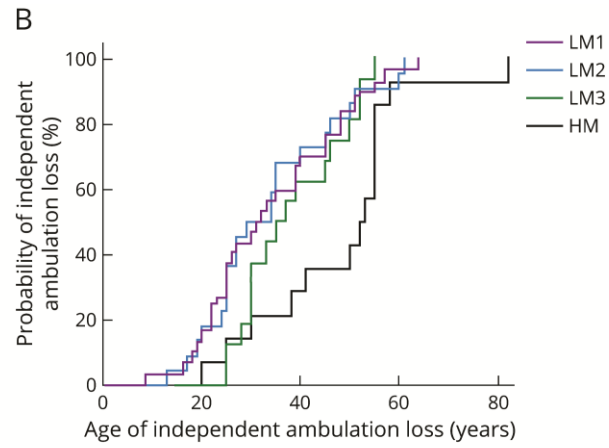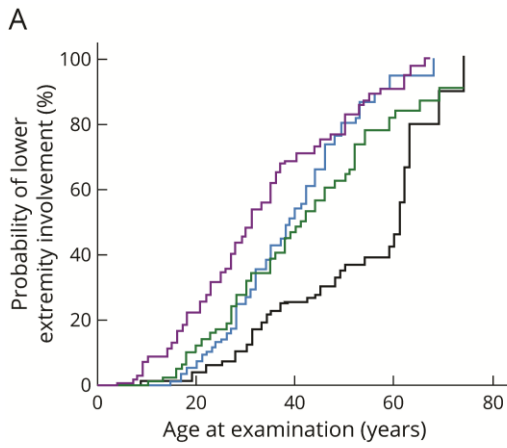
Number of events (%):

—	0 (0)	30 (14.6)	85 (41.3)	104 (50.5)	109 (52.9)
—	0 (0)	8 (3.9)	58 (28.2)	90 (43.7)	91 (44.2)
—	0 (0)	14 (6.8)	50 (24.3)	74 (35.9)	76 (36.9)
—	0 (0)	5 (2.4)	29 (14.1)	40 (19.5)	48 (23.4)

B

Probability of independent ambulation loss (%)

Age of independent ambulation loss (years)

LM1
LM2
LM3
HM

Number of events (%):

—	0 (0)	5 (2.4)	21 (10.2)	29 (14.1)	30 (14.6)
—	0 (0)	4 (1.9)	16 (7.8)	21 (10.2)	22 (10.7)
—	0 (0)	0 (0)	10 (4.9)	16 (7.8)	16 (7.8)
—	0 (0)	1 (0.5)	4 (2.0)	13 (6.3)	13 (6.3)

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