Homozygous nonsense variant in LRIF1 associated with facioscapulohumeral muscular dystrophy

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Abstract

Objective
Facioscapulohumeral muscular dystrophy (FSHD) is a heterogenetic disorder predominantly characterized by progressive facial and scapular muscle weakness. Patients with FSHD either have a contraction of the D4Z4 repeat on chromosome 4q35 or mutations in D4Z4 chromatin modifiers SMCHD1 and DNMT3B, both causing D4Z4 chromatin relaxation and inappropriate expression of the D4Z4-encoded DUX4 gene in skeletal muscle. In this study, we tested the hypothesis whether LRIF1, a known SMCHD1 protein interactor, is a disease gene for idiopathic FSHD2.

Methods
Clinical examination of a patient with idiopathic FSHD2 was combined with pathologic muscle biopsy examination and with genetic, epigenetic, and molecular studies.

Results
A homozygous LRIF1 mutation was identified in a patient with a clinical phenotype consistent with FSHD. This mutation resulted in the absence of the long isoform of LRIF1 protein, D4Z4 chromatin relaxation, and DUX4 and DUX4 target gene expression in myonuclei, all molecular and epigenetic hallmarks of FSHD. In concordance, LRIF1 was shown to bind to the D4Z4 repeat, and knockdown of the LRIF1 long isoform in muscle cells results in DUX4 and DUX4 target gene expression.

Conclusion
LRIF1 is a bona fide disease gene for FSHD2. This study further reinforces the unifying genetic mechanism, which postulates that FSHD is caused by D4Z4 chromatin relaxation, resulting in inappropriate DUX4 expression in skeletal muscle.

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Facioscapulohumeral muscular dystrophy (FSHD; MIM: 158900) is an inherited myopathy in which patients typically have asymmetric weakness of facial, scapular girdle, and upper arm muscles. With disease progression, other muscles may become involved. Most patients (FSHD1) have a contraction of the D4Z4 macrosatellite repeat to a size of 1–10 D4Z4 units on one of their chromosomes 4, whereas European control individuals have 8–~100 units. D4Z4 repeat contractions are associated with partial D4Z4 chromatin relaxation in somatic cells evidenced by, among others, DNA hypomethylation and distinct changes in histone modifications. These epigenetic changes result in expression of the D4Z4-encoded DUX4 (MIM: 606009) retrogene in skeletal muscle. DUX4 lacks a polyadenylation sequence (PAS) in the D4Z4 unit and requires a distally located PAS that is present only in the 4qA haplotype, but not in the 4qB haplotype, nor on chromosome 10, which contains a highly homologous repeat. Hence, patients with FSHD1 have a contracted D4Z4 repeat on the 4qA haplotype. DUX4 encodes for a germline and cleavage-stage double homeobox transcription factor and is toxic when expressed in myogenic cells in vitro and in vivo.

In <5% of patients with FSHD (FSHD2; MIM: 158901), D4Z4 chromatin relaxation occurs in the absence of D4Z4 repeat contraction. Although in FSHD1 cases, D4Z4 hypomethylation only occurs on the contracted allele, in FSHD2, pan-D4Z4 hypomethylation is observed on chromosomes 4 and 10. This suggests that trans-acting factors essential for epigenetic repression of the D4Z4 repeat array are defective. Indeed, in many patients with FSHD2, heterozygous mutations in the structural maintenance of chromosomes flexible hinge domain containing 1 gene (SMCHD1; MIM: 614982), which has a role in epigenetic silencing, are responsible for this pan-D4Z4 hypomethylation. SMCHD1 was reported to compact D4Z4–100 units. D4Z4 repeat contractions are associated with partial D4Z4 chromatin relaxation in somatic cells evidenced by, among others, DNA hypomethylation and distinct changes in histone modifications. These epigenetic changes result in expression of the D4Z4-encoded DUX4 (MIM: 606009) retrogene in skeletal muscle. DUX4 lacks a polyadenylation sequence (PAS) in the D4Z4 unit and requires a distally located PAS that is present only in the 4qA haplotype, but not in the 4qB haplotype, nor on chromosome 10, which contains a highly homologous repeat. Hence, patients with FSHD1 have a contracted D4Z4 repeat on the 4qA haplotype. DUX4 encodes for a germline and cleavage-stage double homeobox transcription factor and is toxic when expressed in myogenic cells in vitro and in vivo.

Methods

Clinical examination of a patient with idiopathic FSHD2 was combined with muscle pathology studies and with genetic, epigenetic, and molecular examinations. Examinations included analysis of D4Z4 methylation by pyrosequencing and Sanger sequencing after bisulfite PCR, Sanger sequencing of LRIF1, whole-exome sequencing, D4Z4 repeat size analysis, Western blot analysis, DUX4 expression analysis, chromatin immunoprecipitation (ChiP)–qPCR analysis, and knockdown experiments. Full methods, and figure e-1 and tables e1–e7, are available on Dryad (doi.org/10.5061/dryad.kwh70rz04).

Results

We previously reported on 20 Japanese patients with FSHD2 showing pan-D4Z4 hypomethylation, of which 13 had an SMCHD1 mutation. We sequenced LRIF1 (GenBank: NM_018372.3) in the remaining 7 patients and found a homozygous duplication variant c.869_872dup in exon 2 in 1 patient, which causes frameshift and leads to a premature stop codon (p.Trp291Ter) (figure 1B). The remaining 6 patients did not show evidence for LRIF1 mutations. Among the 2 LRIF1 protein isoforms, c.869_872dup is predicted to only affect the longer isoform (LRIF1L; figure 1B). Western blot of the immortalized patient’s fibroblasts confirmed the selective absence of LRIF1L (figure 1C). This variant has not been reported in public databases. Subsequent whole-exome sequencing did not identify pathogenic variants in any of the 7 patients in DNMT3B, CAPN3, VCP, FHL1, and FAT1, genes that were previously reported to cause or mimic FSHD when mutated (table e-1, doi.org/10.5061/dryad.kwh70rz04).

The patient, a 53-year-old man born from a consanguineous marriage, experienced difficulty in raising his arms. At age 52 years, he could not walk fast and felt fatigue when climbing stairs. One year later, he had aspiration pneumonia. Muscle weakness of the face, scapular girdle, upper arm, thigh, and neck was noted. The serum creatine kinase level was 89 IU/L. On muscle CT, asymmetric involvement of biceps brachii, quadriceps femoris, gastrocnemius, and paraspinal muscles was documented (figure e-1A, doi.org/10.5061/dryad.kwh70rz04). Muscle pathologic examination identified a few small angular fibers with high alkaline phosphatase enzyme activity (figure e-1B, doi.org/10.5061/dryad.kwh70rz04), which is often seen underlying some patients with FSHD2 remains unresolved, we speculated that SMCHD1 and LRIF1 together ensure a repressed state of the D4Z4 repeat in somatic cells, rendering LRIF1 a candidate gene for idiopathic FSHD2.
Proband exhibits (epi)genetic and molecular characteristics of FSHD

(A) Pedigree of the family. Number of D4Z4 units (U) on chromosome 4, LRIF1 variant status, and D4Z4 DNA methylation as assessed by pyrosequencing from blood DNA are indicated. Alleles with more than 14 D4Z4 units on chromosome 4, which could not be measured by Southern blot are described as >14. (B) Schematic representation of 2 LRIF1 mRNA and protein isoforms with zoom in of Sanger sequencing trace showing homozygous 4 nt duplication (underlined AATG) in exon 2 found in the proband, which leads to a premature stop codon (p.Trp291*). Previously described domains in LRIF1 are also depicted. NLS = nuclear localization signal. Both αLRIF1 antibodies used in this study (Western blot and ChIP) recognize amino acid sequence corresponding to the C-terminus of LRIF1 as indicated by the orange line. (C) Western blot analysis of LRIF1 and SMCHD1 in immortalized fibroblasts from the proband and 2 independent control individuals showing loss of LRIF1L in the proband’s sample, whereas the short isoform (LRIF1S) is still present. SMCHD1 protein levels in the proband were comparable to those in control fibroblasts. Tubulin was used as a loading control. (D) Schematic representation of a D4Z4 repeat unit as a triangle. Regions analyzed for methylation (DR1 site consisting of 19 CpGs, which each can be either methylated—full circle or unmethylated—empty circle) and ChIP-qPCRs (Q) are indicated. Exon 1 of DUX4 is shown as a bar with DUX4 open reading frame depicted with a thick arrow over exon 1. Arrows represent the position of primers used for respective analyses. (E) DNA methylation levels at the D4Z4 DR1 site as assessed by bisulfite PCR, followed by TOPO-TA subcloning from 3 control, 3 FSHD2, and proband immortalized fibroblasts. At least 10 individual colonies were sequenced from each fibroblast line. Bars represent mean methylation ±SEM of all CpGs present in the amplicon from all analyzed colonies. (F) ChIP for histone modifications (H3K9me3, H3K27me3, and H3K4me2) was performed in 3 control, 2 FSHD2, and proband immortalized fibroblasts, followed by qPCR with primers specific for the Q region in D4Z4. Normal anti-rabbit immunoglobulin G was used as a negative control. Data represent the ChIP enrichment relative to input and normalized to H3 enrichment. The error bars represent mean ±SEM. (G) Expression analysis of DUX4 and its 2 transcription target genes (ZSCAN4 and TRIM43) expression in myotubes using RT-qPCR. Immortalized fibroblasts were transduced with lentivirus carrying either MYOD1 induce transdifferentiation toward myogenic lineage or 3xFlag as a negative control. DUX4 and its target genes expression level was normalized to the GUSB expression level. Each sample was analyzed in biological duplicate. The error bars indicate mean ± SD. ChIP = chromatin immunoprecipitation; FSHD = Facioscapulohumeral muscular dystrophy; qPCR = quantitative polymerase chain reaction.
in FSHD. Comparison of the clinical phenotype and severity of the proband with 13 patients with FSHD2 carrying SMCHD1 variants from our previous study\(^\text{10}\) did not reveal any obvious difference (tables e-2 and e-3 and figure e-1C, doi.org/10.5061/dryad.kwh70rz04).

In addition to the LRIF1 variant, the proband carries a D4Z4 repeat of 13 units on a 4qA haplotype (figure e-1D, doi.org/10.5061/dryad.kwh70rz04), consistent with the digenic inheritance of FSHD2 in which a combination of a D4Z4 chromatin factor mutation and an FSHD-permissive chromosome 4 causes disease.

D4Z4 methylation in the proband’s blood was 15% as determined by bisulfite pyrosequencing (normal range >25%). D4Z4 methylation of the healthy mother, who is a heterozygous mutation carrier, was within the normal range (60%; figure 1A). D4Z4 chromatin relaxation (figure 1D) was confirmed in patient immortalized fibroblasts, showing reduced D4Z4 DNA methylation (figure 1E and e-1E, doi.org/10.5061/dryad.kwh70rz04), a partial loss of H3K9me3, and a gain of H3K4me2 and H3K27me3 at D4Z4 (figure 1F). These D4Z4 chromatin changes are typically found in FSHD2.\(^\text{1,8}\)

The molecular hallmark of FSHD is the expression of DUX4 in myotubes. MYOD1-mediated transdifferentiation of immortalized skin fibroblasts of the proband into myotubes, as evidenced by increased expression of MYOG and MYH3 (figure e-1F, doi.org/10.5061/dryad.kwh70rz04), resulted in DUX4 and DUX4 target gene expression at levels comparable to FSHD1 myotubes, confirming that the epigenetic changes of D4Z4 observed in the patient correlate with transcriptional derepression of this locus (figure 1G).

Consistent with earlier data,\(^\text{7}\) LRIF1 interacts with SMCHD1 when coexpressed (figure e-1G, doi.org/10.5061/dryad.kwh70rz04). ChIP studies showed that LRIF1 binds to D4Z4 repeats in control primary myoblasts and myotubes (figure 2, A and B), as does SMCHD1, suggesting that both proteins function together in repressing D4Z4 in muscle tissue. In the proband’s immortalized fibroblasts, we observed reduced enrichment of LRIF1 and of SMCHD1 at D4Z4 compared with

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**Figure 2** LRIF1 binds to D4Z4 in myogenic cells, and its binding is together with SMCHD1 reduced in the proband

(A) ChIP was performed with antibodies specific for SMCHD1, LRIF1, or normal anti-rabbit IgG (negative control) in 4 control primary myoblasts, followed by qPCR with primers specific for either the Q region or the GAPDH promoter region, which served as a negative control region for SMCHD1 and LRIF1 enrichment. Data represent the ChIP values as relative to input in %. Bars represent the mean ± SEM. (B) ChIP was performed as in (A) but with 4 control primary myoblasts after their differentiation to myotubes. (C) LRIF1 ChIP-qPCR for the Q region of D4Z4 from 3 control immortalized fibroblasts and the proband’s fibroblasts showing reduced LRIF1 enrichment at this region in proband compared with control fibroblasts. Bars represent the mean ± SEM, and enrichment is shown as relative to input in %. (D) SMCHD1 ChIP-qPCR for the Q region of D4Z4 in 3 control, 4 FSHD2, and proband immortalized fibroblasts showing reduced SMCHD1 binding to this region in proband in comparison to control fibroblasts as observed also for FSHD2 samples. ChIP = chromatin immunoprecipitation.
control immortalized fibroblasts (figure 2, C and D) with the SMCHD1 enrichment in the proband being comparable to that in FSHD2 immortalized fibroblasts, despite the normal SMCHD1 protein levels in the proband (figure 1C).

To confirm that the loss of the LRIF1L leads to DUX4 derepression in myogenic cells, we performed siRNA knockdown experiments in control, FSHD1, and FSHD2 immortalized myoblasts. When sufficiently reduced, as confirmed by Western blot analysis, 2 of 3 independent siRNA-mediated knockdowns of LRIF1L resulted in DUX4 derepression in all genetic situations, along with the transcriptional upregulation of well-established DUX4 biomarkers (figure 3).

**Discussion**

This study identifies LRIF1 as an FSHD2 disease gene in a patient having a phenotype that is consistent with idiopathic FSHD. LRIF1 mutations are, like DNMT3B mutations, likely a rare cause of FSHD and should only be considered in FSHD2 when tested negative for SMCHD1 mutations. Of interest, although almost all...
patients with FSHD2 show monoallelic mutations in SMCHD1 or DNMT3B, this patient has a biallelic LRIF1 mutation. This may suggest that a complete loss of full-length LRIF1 is required to derepress DUX4 in skeletal muscle and to cause disease. By showing its involvement in D4Z4 chromatin regulation, like the previously identified FSHD2 disease genes SMCHD1 and DNMT3B, this study reinforces the uniform disease mechanism for FSHD that postulates that the disease is caused by inappropriate expression of DUX4 in skeletal muscle as a result of partial chromatin relaxation of the D4Z4 repeat. Patients with FSHD2 should therefore equally qualify for current and future therapeutic trials targeting DUX4 expression or function.

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Disclosure
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Appendix

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References

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