

ARTICLE

Identification of two novel *SMCHD1* sequence variants in families with FSHD-like muscular dystrophy

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Facioscapulohumeral muscular dystrophy 1 (FSHD1) is caused by a contraction in the number of *D4Z4* repeats on chromosome 4, resulting in relaxation of *D4Z4* chromatin causing inappropriate expression of *DUX4* in skeletal muscle. Clinical severity is inversely related to the number of repeats. In contrast, FSHD2 patients also have inappropriate expression of *DUX4* in skeletal muscle, but due to constitutional mutations in *SMCHD1* (structural maintenance of chromosomes flexible hinge domain containing 1), which cause global hypomethylation and hence general relaxation of chromatin. Thirty patients originally referred for FSHD testing were screened for *SMCHD1* mutations. Twenty-nine had > 11 *D4Z4* repeats. *SMCHD1* c.1040 + 1G > A, a pathogenic splice-site variant, was identified in a FSHD1 family with a borderline number of *D4Z4* repeats (10) and a variable phenotype (in which a *LMNA1* sequence variant was previously described), and *SMCHD1* c.2606 G > T, a putative missense variant (p.Gly869Val) with strong *in vitro* indications of pathogenicity, was identified in a family with an unusual muscular dystrophy with some FSHD-like features. The two families described here emphasise the genetic complexity of muscular dystrophies. As *SMCHD1* has a wider role in global genomic methylation, the possibility exists that it could be involved in other complex undiagnosed muscle disorders. Thus far, only 15 constitutional mutations have been identified in *SMCHD1*, and these two sequence variants add to the molecular and phenotypic spectrum associated with FSHD.

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INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common muscular dystrophy after the dystrophinopathies and myotonic dystrophy.^{1–3} It is inherited in an autosomal dominant manner, affecting about 1 in 20 000 individuals worldwide.⁴ FSHD is characterised by progressive muscle weakness involving atrophy of the muscles of the face, upper arm and shoulder girdle. General muscle weakness and atrophy may eventually involve the musculature of the pelvic girdle and foot extensors. Early onset of the disease is usually associated with the development of the most severe forms of the disorder.⁴ Disease onset is unusual before the age of 10 and the disease is usually penetrant by age 30. The majority of patients develop symptoms during the second decade of life. Both retinal vasculopathy and high-tone deafness may be seen as a part of FSHD.

The *FSHD1* locus accounts for 95% of clinical disease and maps to 4q35.⁵ FSHD1 patients carry a large deletion in the polymorphic *D4Z4* macrosatellite repeat array at 4q35 and present with 1–11 repeats whereas non-affected individuals possess 12–150 repeats. An almost identical repeat array is present at 10q26; the high sequence identity between these two arrays has proved to be challenging for molecular diagnosis. Each 3.3-kb *D4Z4* unit contains a *DUX4* (double homeobox 4) gene that, among others, is activated on contraction of the 4q35 repeat array due to the induction of chromatin remodelling of the 4qter region. A number of 4q subtelomeric sequence variants are now recognised, although FSHD1 only occurs in association with 'permissive' haplotypes, each of which is associated with a polyadenylation signal located immediately distal of the last *D4Z4* unit.^{6,7} The resulting poly-A tail appears to stabilise *DUX4* mRNAs transcribed from this most distal *D4Z4* unit in FSHD muscle cells.

Synthesis of both the *DUX4* transcripts and the protein in FSHD muscle cells induces significant cell toxicity.⁸ *DUX4* is a transcription factor that may target several genes that results in a deregulation cascade, which, in turn, inhibits myogenesis, sensitises cells to oxidative stress and induces muscle atrophy, thus epitomising many important molecular features of FSHD.

DUX4 is normally expressed in the germline,^{9,10} whereas it is epigenetically repressed in healthy somatic cells including differentiated muscle tissue. FSHD1 is caused by the contraction of *D4Z4* array that results in DNA hypomethylation and decreased repressive heterochromatin (chromatin relaxation) in the 4q35 region, leading to ectopic *DUX4* expression.

Approximately 5% of FSHD patients do not have a contraction in the *D4Z4* array, and were historically ascribed to a putative FSHD2 locus. Whole-exome sequencing identified the *SMCHD1* gene as the FSHD2 locus.¹¹ The function of the *SMCHD1* protein is to aid in the methylation of large chromosomal regions, including the X chromosome and the *D4Z4* array. However, not all FSHD2 patients could be explained by contraction of the *D4Z4* repeats or mutations in *SMCHD1*, implying that an FSHD3 locus exists.

Loss of *SMCHD1* activity in FSHD2 patients reduces methylation of the *D4Z4* array, allowing the genetic transcription machinery to gain access to the *DUX4* gene.¹¹ FSHD2 is caused by co-inheritance of two independent events: an FSHD-permissive chromosome 4 haplotype (necessary for polyadenylation of *DUX4* mRNA) and a variant in *SMCHD1* that causes *D4Z4* hypomethylation, neither of which by itself causes disease.¹¹ The *SMCHD1* gene product codes for protein that regulates chromatin repression in a wide variety of organisms.^{10,12} Given the wider role of *SMCHD1* in regulating

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methylation, it is possible that *SMCHD1* could be a modifier in human diseases.

In this study, we screened 30 patients with FSHD or FSHD-like features, of whom 29 did not exhibit a contraction of *D4Z4* and 1 a borderline *D4Z4* repeat contraction (10 repeats).

MATERIALS AND METHODS

High molecular weight DNA was available from 30 FSHD patients¹³ who had been referred to our centre for either research or diagnostic testing. All patients had previously undergone DNA diagnostic testing for FSHD1, and all but one had been identified as carrying >11 4q35-located *D4Z4* repeats, as determined by p13E11 hybridisation to *EcoRI*-digested and *EcoRI/BlnI*-digested genomic DNA.^{13,14} The 4qA-defined and 4qB-defined subtelomeric alleles were also identified as previously reported.^{13,14} 4q35.2-associated haplotypes were assessed as previously reported.^{15,16}

D4Z4 methylation analysis

Methylation-sensitive restriction digestion. The methylation level in the 4q-associated proximal *D4Z4* tandem repeat was determined using the methylation-sensitive enzyme *FseI*.¹¹ Following blotting the band intensities were quantified using ImageJ software (v1.43, National Institutes of Health, Bethesda, MD, USA). Some of the samples were also analysed by pyrosequencing of DR1 region of *DUX4*.¹⁷

RNA analysis

RNA was extracted from lymphocytes and was reverse transcribed to complementary DNA (cDNA). Five microliters of 5 ng/μl cDNA was amplified using 40 cycles and sequenced using standard methods.¹¹ Location of primers used is given in Supplementary Table 1.

Bioinformatic analysis

The disease-causing potential of the missense mutation (NM_015295.2:c.2606G>T: p.Gly869Val) was evaluated using Condel (<http://bg.upf.edu/condel/home>).¹⁸ The output of Condel is based on the consensus of *in silico* predictions from SIFT (<http://sift.jcvi.org>),¹⁹ Polyphen2 (<http://genetics.bwh.harvard.edu/pph2>)²⁰ and Mutation Assessor (<http://mutationassessor.org>).²¹ Condel is a computational method used to assess whether a missense variant results in an amino-acid substitution that is neutral or deleterious to the respective protein. In addition, evolutionary sequence conservation was assessed using a multiple sequence alignment of 13 orthologous proteins. The assessment of the potential impact of p.Gly869Val on splicing was conducted using MutPred Splice (<http://mutdb.org/mutpredsplice>)²² and by computing the net change to exonic regulatory elements, for example, loss of exonic splicing enhancers (ESE) and/or gain of exonic splicing silencers (ESS), which may serve to weaken exon definition and promote exon skipping. The set of ESE and ESS motifs considered in this analysis are derived from the NI-ESE and NI-ESS set.

RESULTS

Clinical details

The pedigree of family A and B are shown in Figures 1a and 2a, respectively. Individual clinical findings are summarised in Table 1. Detailed clinical findings are described in the Supplementary Material.

Molecular genetic and bioinformatic analysis

DNA sequencing of *SMCHD1* in 30 FSHD patients exhibiting facial muscle weakness and weakness of either upper limb or lower limb or both revealed a novel obligatory donor splice-site variant c.1040+1G>A in FSHD1 family A (Figure 1b), segregating with disease. All the affected individuals in this family carry 10 units of *D4Z4* array. This novel splice-site variant has occurred against the permissive background for FSHD1.¹¹ It was predicted *in silico* to abolish the 5' splice site (wt = 0.98, mut = 0), and as RNA analysis did

not reveal any exon skipping, it can be concluded that nonsense-mediated decay (NMD) had nullified production of mRNA from the mutant allele (Figure 1c), although in the absence of a normal control this test can only be considered to be semi-quantitative. In addition, methylation at *D4Z4* loci was observed in all of the affected individuals (17, 16 and 16%) but not in the unaffected (31%) (Figure 1d).

In family B, owing to the limited amount of DNA available from this family, 4qA analysis was not done but SSLP analysis generated 163 and 166 bp alleles in each of the three sisters, which are unlikely to be on the permissive allele.¹⁶ In this family, a novel missense variant, c.2606 G>T, p.Gly869Val was identified in the *SMCHD1* gene (Figure 2b). The bioinformatic analysis of p.Gly869Val using Condel predicted this variant to be pathogenic (Condel score = 0.896).¹⁸

By deriving a prediction based on the consensus of these tools, Condel outperforms each method when applied individually. It has been estimated that Condel achieves an accuracy of ~88–89% in classifying the impact of a missense variant as neutral or deleterious. A computational method should not be used alone to identify a disease-causing variant, but does give supporting evidence when accompanied by other lines of evidence (for example, segregation and *in vitro/in vivo* functional studies). In addition, the residue where the variant occurs (p.Gly869) is highly conserved (100%) based on the multiple sequence alignment of 13 orthologous proteins investigated here (Figure 2e). This variant (c.2606G>T) is predicted to disrupt splicing with MutPred Splice.²² If this change was responsible for exon skipping (in a proportion of transcripts), this would cause a frame shift of the reading frame, resulting in a transcript with a premature termination codon (PTC). This mis-spliced transcript containing the PTC may therefore be subject to NMD. The limitation of our RT-PCR assay is that in the absence of a reference gene, it is semi-quantitative (Figure 2c). It is also likely that any pathogenic effect of p.Gly869Val is due to the impact of this amino-acid substitution at the protein level. Due to restricted DNA sample from II-1 and II-2 in family B, DNA samples were analysed by pyrosequencing in another clinically accredited diagnostic laboratory.¹⁷ DNA samples from II.2 and II.3 with sequence variant also revealed borderline hypomethylation (37%) based on cutoff being 40% (Debbie Smith, personal communication) whereas II-1 lacked hypomethylation (53%) (Figure 2d).

DISCUSSION

The underlying genetic cause of FSHD has proven singularly difficult to elucidate. It took 21 years following the mapping of the disease to 4q35, to find the underlying genetic cause of FSHD1^{6,7} and two years later, exome sequencing has revealed that mutations in the *SMCHD1* gene are the cause of FSHD2.¹¹ To date, only 15 constitutional mutations causative of FSHD2 have been identified in the *SMCHD1* gene.^{11,23} Additional two variants from the current study will add to the molecular and phenotypic spectrum associated with FSHD.

Affected members of family A have a phenotype that varies between individuals, but is generally of later onset than classical FSHD (Figure 3). All affected individuals carry 10 *D4Z4* repeats on the FSHD-permissive haplotype and also have the pathogenic splice-site variant *SMCHD1* c.1040+1G>A.

Clinically, FSHD1 cannot be distinguished from FSHD2.²⁴ Although FSHD1 and FSHD2 have different underlying genetic defects, they both appear to be caused by transcriptional derepression of *DUX4* in skeletal muscle.¹¹ The contraction of *D4Z4* repeats in FSHD1 is associated with partial demethylation of the shortened allele, and relaxation of the local chromatin structure, whereas, in FSHD2, global genomic demethylation is observed.

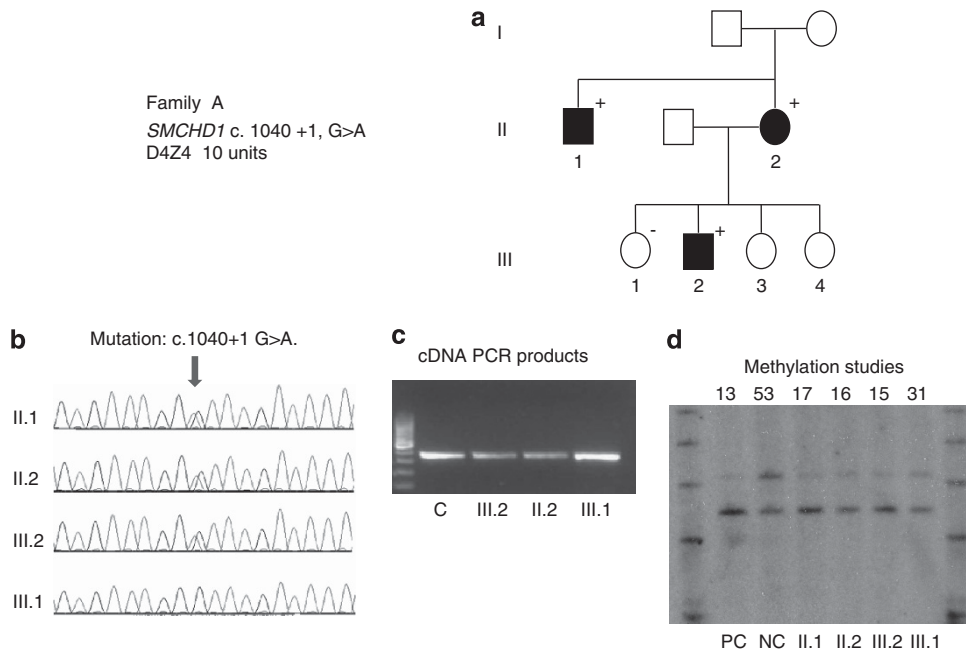


Figure 1 Genetic and epigenetic characterisation of family A. (a) Pedigree. Shaded boxes represent affected individuals. + and – denote the presence and absence of variant. (b) Mutation analysis by Sanger sequencing. (c) Reverse transcription-PCR (RT-PCR): the agarose gel images show RT-PCR fragments from the affected individuals. (d) Methylation analysis: DNA digested with *EcoRI* and *FseI* and hybridised with probe p13E11. Percent methylation for each sample is shown above each lane.

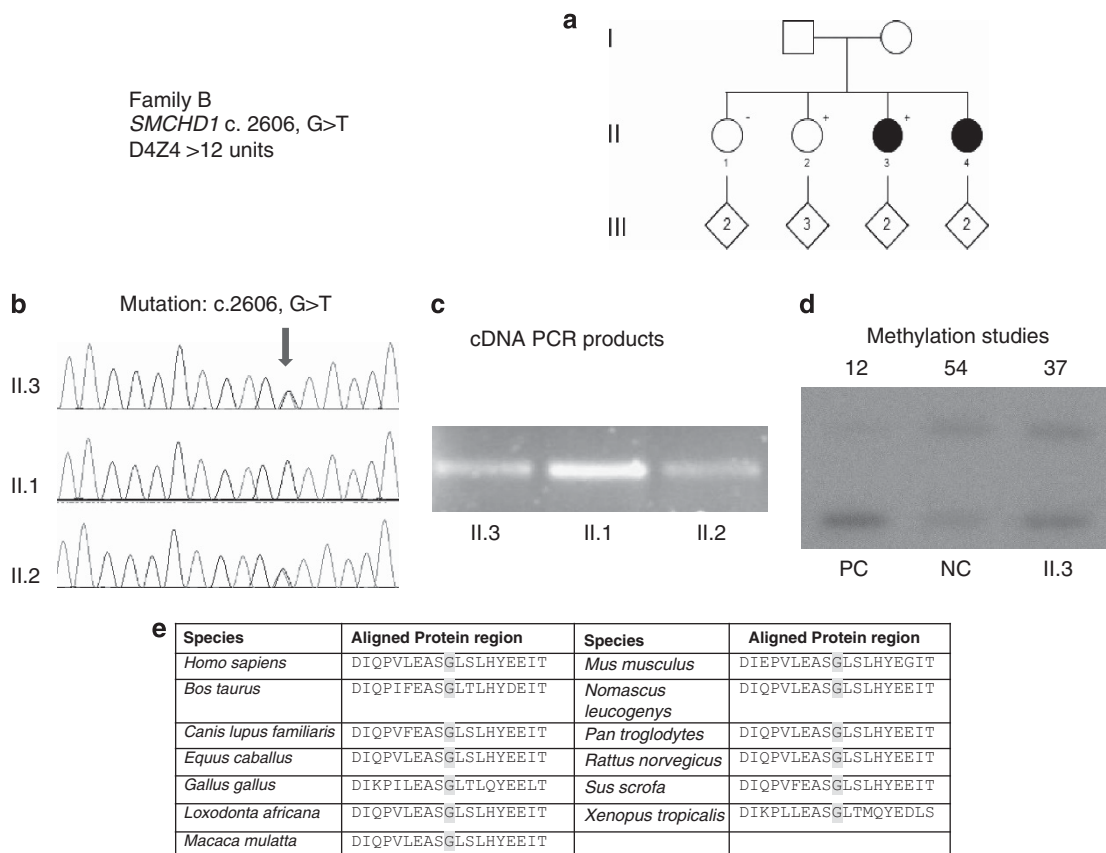


Figure 2 Genetic and epigenetic characterisation of family B. (a) Pedigree. Shaded boxes represent affected individuals. + and – denote the presence and absence of variant. (b) Mutation analysis by Sanger sequencing. (c) Reverse transcription-PCR (RT-PCR): the agarose gel images show RT-PCR fragments from the affected individuals. (d) Methylation analysis: DNA digested with *EcoRI* and *FseI* and hybridised with probe p13E11. Percent methylation for each sample is shown above each lane. (e) Multiple sequence alignment of 13 orthologous protein.

Table 1 Clinical and molecular findings of the members of family A and B

Family	Individual	Gender	Phenotype	Age of onset/ presentation	SMCHD1 genotype (ref. sequence- NM_015295.2)	SMCHD1 genotype (ref. sequence- NG_031972.1)	RNA analysis	D4Z4 repeats	D4Z4 methylation	
A	II:1	M	FSHD, late onset	Early 50s	c.1040 + 1G>A	g.43807 G>A	r.(0)	10	17%	
	II:2	F	Erb's juvenile dystrophy	24	c.1040 + 1G>A	g.43807 G>A	r.(0)	10	16%	
			Peroneal muscular atrophy	27						
			LGMD	29						
			FSHD, adult onset	>> 29						
III:1	F	Unaffected	42	c.1040 + 1G	g.43807 G	r.(=)		53%		
III:2	M	FSHD, late onset	34	c.1040 + 1G>A	g.43807 G>A	r.(0)	10	16%		
B	II:1	F	Unaffected		c.2606G	g.74014 G	r.(=)	Unknown	52% (pyrosequencing)	
	II:2	F	Unaffected		c.2606G>T	g.74014 G>T	r.(0)	> 12	37% (pyrosequencing)	
	II:3	F	Affected	41	c.2606G>T	g.74014G>T	r.(0)	> 12	37% (pyro and southern blot)	

Abbreviations: F, female; FSHD, facioscapulohumeral muscular dystrophy; LGMD, Limb-Girdle Muscular Dystrophy; M, male.

Transcript reference sequence for *SMCHD1*: NM_015295.2. Genomic reference sequence for *SMCHD1*: NG_031972.1. Data have been submitted to the *SMCHD1* gene variant database at <http://www.LOVD.nl/SMCHD1>, patient IDs 0032035 and 0032036.

Family A
Proband
Individual II:1



Elbow contracture and biceps wasting, 'round shouldered' appearance consistent with shouldered girdle weakness

Winging of scapulae, wasting of humeral muscles, no facial weakness

Sister (II:2) with similar problems – "CMT", cardiac pacemaker for heart block

Figure 3 Family A proband II.1.

Hence, given this common mechanism it is reasonable to suppose that combined FSHD1- and FSHD2-associated genetic changes may be instrumental in the development of FSHD. Three scenarios are thus suggested by family A:

1. The individuals are affected primarily by their borderline short E/B fragment (10 *D4Z4* units), with the *SMCHD1* sequence variant acting as a modifier,
2. the *SMCHD1* sequence variant is primarily causative of their condition and the borderline short E/B fragment is acting as a modifier or
3. both the borderline short E/B fragment and *SMCHD1* sequence variant are acting equally to cause their disease.²³

It is quite possible that other families of this nature and even different individuals within them may be best described by any of the

three scenarios. This will only become clear with the study of more individuals and families, and so we would wish to encourage this.

Family A is of additional interest because it has been reported to have a sequence variant in the *LMNA* gene in association with a remarkably variable phenotype but without perfect segregation of the mutation with the phenotype.²⁵ It is now clear that the *LMNA* mutation is an innocuous polymorphism. This illustrates the complexity of elucidating the underlying cause of genetic conditions and muscle disorders in particular, which is reflected by our finding of a *SMCHD1* sequence variant in the affected individuals.

In family B, II.3 (Figure 4) has an undiagnosed muscular dystrophy with an unusual phenotype including some FSHD-like features (facial muscle weakness) and hence why FSHD diagnostic testing was originally requested. However, this individual does not have a short *D4Z4* repeat array of the type associated with FSHD1. Results on SSLP analysis identified alleles 163 and 166 in all the three sisters but because of restricted amount of DNA, 4qA analysis was not done. SSLP results suggest that *SMCHD1* sequence variant may not have occurred on the permissive background. However, she and her sister (II-2) with sequence change reveal borderline hypomethylation at *D4Z4*, which would be more compatible with FSHD1 rather than FSHD2. Individual II.4 has not been clinically/molecularly assessed as she is not in the country. The DNA samples from either generation 1 or 3 were not available in this family. FSHD was never considered a likely diagnosis in this family, but testing of FSHD was undertaken to exclude this as a potential confounder. It was intriguing, therefore, to find a novel putative missense change in *SMCHD1* (c.2606 G>T, p.Gly869Val) in II.3, which does not appear to segregate with the disease in the family; however, it is possible that II.2 with the variant in this family is non-penetrant for the disease. Bioinformatic analysis strongly suggests the variant to be pathogenic. The amino-acid residue corresponding to human *SMCHD1* p.Gly869 is highly conserved in evolution across 13 species, thus implying that it is of functional importance. For confirmation of FSHD, accurate results determining the genetic background on chromosome 4 are necessary in sporadic cases and in patients presenting with an atypical clinical phenotype. Mutations in other genes may also mimic FSHD, including mutations in *CAPN3*, *VCP* and *FHL1*.^{26–28} Intriguingly, *SMCHD1* c.3651A>G, a missense variant (p.Ile1217Met), has been reported in a family with autistic spectrum disorder,²⁹ which shows that this gene may also have a role to play in non-muscle disorders.



Family B proband II:3

- Relative paucity of facial expression and mild myopathic facies
- Note absence of true ptosis, but dermatochalasis (excess redundant skin around the upper eye)
- Mild dysmorphism with low-set, simple, cupped ears
- Small jaw

Figure 4 Family B proband II.3.

Hence, although family B does not have classical FSHD1 or FSHD2, clinically or molecularly, the possibility exists that *SMCHD1* sequence variant may be involved in the pathogenesis of other myopathies and because *SMCHD1* has a wider role in global genomic methylation the possibility exists that it could be involved in other complex undiagnosed muscle disorders. This may, of course, be an incidental finding, but certainly suggests further studies defining the role of *SMCHD1* in FSHD-associated conditions or other dystrophies are warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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