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SHORT REPORT

Characterization of *D4Z4* alleles and assessment of de novo cases in Facioscapulohumeral dystrophy (FSHD) in a cohort of Italian families

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Abstract

Facioscapulohumeral dystrophy (FSHD) is an autosomal dominant disease, although 10%–30% of cases are sporadic. However, this percentage may include truly de novo patients (carrying a reduced *D4Z4* allele that is not present in either of the parents) and patients with apparently sporadic disease resulting from mosaicism, non-penetrance, or complex genetic situations in either patients or parents. In this study, we characterized the *D4Z4* Reduced Alleles (DRA) and evaluated the frequency of truly de novo cases in FSHD1 in a cohort of DNA samples received consecutively for FSHD-diagnostic from 100 Italian families. The *D4Z4* testing revealed that 60 families reported a DRA compatible with FSHD1 (1–10 RU). The DRA co-segregated with the disease in most cases. Five families with truly de novo cases were identified, suggesting that this condition may be slightly lower (8%) than previously reported. In addition, *D4Z4* characterization in the investigated families showed 4% of mosaic cases and 2% with translocations. This study further highlighted the importance of performing family studies for clarifying apparently sporadic FSHD cases, with significant

implications for genetic counseling, diagnosis, clinical management, and procreative choices for patients and families.

KEYWORDS
D4Z4, de novo patients, family study, FSHD, genetic counseling

1 | INTRODUCTION

Facioscapulohumeral dystrophy (FSHD) is a genetic disease characterized by a progressive skeletal muscle weakness affecting facial, upper, and lower extremities.[1] Although the disease typically displays an autosomal dominant inheritance, reduced penetrance and variable expressivity complicate the diagnosis and the genotype–phenotype correlation in single cases as well as within families. The genetic cause of FSHD has been associated with the alteration of the D4Z4 region on chromosome 4q35, that normally consists of 11–100 Repeated Units (RU) and it is subjected to epigenetic repression. Two subtelomeric variants (4qA and 4qB) have been identified at chromosome 4, although only the 4qA is associated with FSHD and it is referred to as "permissive" allele.[2] Two forms of FSHD are traditionally known, namely FSHD1 and FSHD2. The first form is caused by the partial deletion of the number of RUs within the *D4Z4*, which is reduced to 1–10 RUs. The second form is generally characterized by longer *D4Z4* size (up to 20 RUs) in combination with detrimental variants within *SMCHD1*, *DNMT3B*, and *LRIF1* genes, which are involved in the epigenetic repression of the locus.[3–6] Importantly, variants in FSHD2 genes can also act as disease modifiers in the presence of *D4Z4* Reduced Allele (DRA) < 10 RU.[4,6]

The estimated prevalence of FSHD ranges from 1:8000 to 1:20.000.[1,7] Although the mode of inheritance is autosomal dominant, 10%–30% of cases are sporadic.[8,9] Several Mendelian disorders show approximately 7%–10% of sporadic cases explained by de novo genetic alterations, while other conditions (Duchenne Muscular Dystrophy) reports higher rates (33%).[10,11]

Concerning FSHD, the percentage of sporadic cases may not represent the real frequency of truly de novo cases, that is, patients carrying a DRA that is not present in either of the parents.[12]

This percentage could in fact include not only de novo patients but also subjects with apparently sporadic disease deriving from complex genetic rearrangements, such as 4q/10q translocations, in the parents as well as patients who inherited the DRA from an asymptomatic (non-penetrant) parent carrier of the short allele. Previous works highlighted that up to 20%–40% of apparently sporadic FSHD1 cases could be explained by mosaicism in unaffected parents or patients.[8,13] Given these premises, we took advantage of our experience as Italian Reference Centre for FSHD diagnosis and the availability of 2550 patients' samples to characterize the *D4Z4* alleles and evaluate the frequency of truly de novo FSHD1 cases in the Italian population.

2 | MATERIALS AND METHODS

The study relied on the availability of a large cohort of samples who accessed the laboratory of Medical Genomics-UILDM at the Santa Lucia Foundation (Rome) for FSHD genetic testing, from 2000 to 2022. Only families in which both parents were available and the familiarity was ascertained were included in the study. As a result, a cohort of consecutive 100 families (354 subjects in total) fulfilled these criteria. The study cohort had an average age of 50 ± 17 years and a male:female (M:F) ratio of 57:43. The presence of DRA was tested using Pulsed-Field Gel Electrophoresis (PFGE) and Southern blotting followed by hybridization with P13-E11 probe as previously described.[14] The characterization of 4qA and 4qB allelic variants was performed by an additional hybridization with specific probes according to standard procedures. The number of RU was estimated considering the size of the EcoRI fragments (kb) and the formula reported below:

$$\text{Number of Repeated Units} = \frac{[\text{EcoRI fragment length (kb)} - (8.6\,\text{kb})]}{3.3\,\text{kb}}.$$

The formula was derived from Salani et al.[15] For FSHD1, the threshold for assessing the presence of DRA was fixed at *D4Z4* size ≤43 kb, which corresponds to ≤10 RU. However, patients reporting *D4Z4* size ranging from 11 and 20 RU were also reported, because this size range is usually found in FSHD2 cases.

3 | RESULTS AND DISCUSSION

The study allowed identifying a DRA (≤10 RU) compatible with FSHD1 in 60 families (94%) out of 100 and a variant in *SMCHD1* combined with 8–10 RU (i.e., FSHD2) in four families (6%). In addition, the study highlighted 11 families with affected subjects carrying *D4Z4* alleles ranging from 11 and 20 RU. These patients could thereby fall within the FSHD spectrum,[16] although a variant in a causative gene has not yet been identified. All *D4Z4* alleles compatible with FSHD were confirmed to be permissive (i.e., 4qA) for the disease, except for four unaffected subjects who displayed non-pathogenic (i.e., 4qB) alleles ≤20 RU.

Among subjects carrying a DRA, a higher proportion of patients were males (61%) compared to females (39%) and presented an average age of 49 ± 21 and 51 ± 19 years, respectively.

Among the families positive for the FSHD1 genetic testing, the DRA co-segregated with the disease in most cases, except for seven

TABLE 1 Molecular characteristics of families harboring de novo FSHD cases.

ID family	ID subject	Sex	Age	Status	*D4Z4* size (kb)	RU	4qA/4qB	Notes
FSHD19	II:1	M	22	Affected	23	4	A/A	Mosaicism
	I:1	M	66	Unaffected	>75	>20	A/A	
	I:2	F	58	Unaffected	>75	>20	A/A	
FSHD20	II:3	F	26	Affected	10	1	A/B	
	I:2	F	61	Unaffected	>75	>20	B/B	
	I:1	M	61	Unaffected	>75	>20	A/A	
	II:1	F	41	Unaffected	>75	>20	A/A	
	II:2	M	42	Unaffected	>75	>20	A/B	
FSHD28	II:1	M	57	Affected	13	1	A/B	
	I:2	F	86	Unaffected	>75	>20	A/A	
	I:1	M	88	Unaffected	>75	>20	A/B	
FSHD31	II:2	F	64	Affected	18	3	A/B	
	I:1	M	99	Unaffected	>75	>20	A/B	
	I:2	F	93	Unaffected	>75	>20	A/B	
FSHD42	II:1	F	33	Affected	18	3	A/B	
	I:2	F	54	Unaffected	>75	>20	A/B	
	I:1	M	59	Unaffected	>75	>20	A/A	
FSHD52	II:1	M	24	Affected	20	3	A/A	Multiple translocations 4q/10q
	II:2	M	19	Affected	20	3	A/A	Mosaicism
	I:1	M	53	Unaffected	>75	>20	A/A	
	I:2	F	50	Unaffected	>75	>20	A/A	
FSHD79	II:1	M	56	Affected	25	5	A/B	
	I:2	F	79	Unaffected	>75	>20	A/B	
	I:1	M	83	Unaffected	>75	>20	A/A	
	II:2	F	53	Unaffected	>75	>20	A/B	

Note: The *D4Z4* size column reports the shortest 4qA allele compatible with FSHD.
Abbreviations: kb, kilobases; RU, repeated unit.

families. In these cases, the DRA was detected only in the proband, with both unaffected parents carrying normal *D4Z4* alleles (Table 1). The phenotype of patients was evaluated by expert neurologists and was consistent with FSHD in terms of disease onset, localization of muscle weakness and severity.[1,17]

Consistent with this result, the probands were sporadic cases in families without a history of FSHD, thus, suggesting they could represent de novo cases. However, a sporadic patient (FSHD19_II:1) was found to be a carrier of somatic mosaicism (defined by a fifth fragment of reduced density hybridizing with p13E-11 and visualized on PFGE), whereas two subjects (FSHD52_II:1 and FSHD52_II:2) of another family were shown to carry multiple 4q/10q translocations and somatic mosaicism for the disease allele, respectively (Figure S1). The latter cases had parents with apparently normal sized *D4Z4* alleles, although the presence of complex rearrangements or germinal mosaicisms undetectable by PFGE cannot be excluded. On this subject, advanced technologies (molecular combing, nanopore, and Optical Genome Mapping) may clarify this case, given the higher resolution and precision in detecting complex rearrangements. In the remaining five families (FSHD20, FSHD28, FSHD31, FSHD42, and FSHD79), the 4q and 10q alleles showed a standard configuration in patients carrying the DRA, confirming them as truly de novo FSHD cases. These results showed a slightly lower percentage of truly de novo FSHD1 cases (8%) with respect to the minimum value (10%) previously reported.[9] In addition, most (4/5) of truly de novo cases displayed DRA ranging from 1 to 3 RU, that is consistent with a previous study highlighting that the 60.6% of 1–3 RU carriers were de novo cases.[12] Furthermore, the study identified 20 unaffected subjects carrying a DRA ≤ 10 RU (Table S1). Of them, one subject (FSHD18_I:2) carried a short 4qB allele, which is not permissive for FSHD, whereas the other individuals displayed a short 4qA allele <10 RU, representing thereby non-penetrant gene carriers. Interestingly, most of them (18/20) were females, in line with previous studies reporting a higher proportion of women among the asymptomatic/unaffected carriers.[8–19] The non-penetrant gene carriers showed an average age of 60 years, except for 3 younger (<35 years of age) subjects (FSHD51_II:2, FSHD68_II:1, and FSHD100_II:2) that should be monitored to rule out the manifestation of FSHD later in life.

In addition, the study highlighted the presence of mosaicism with a short DRA in four families (FSHD13, FSHD14, FSHD19, and

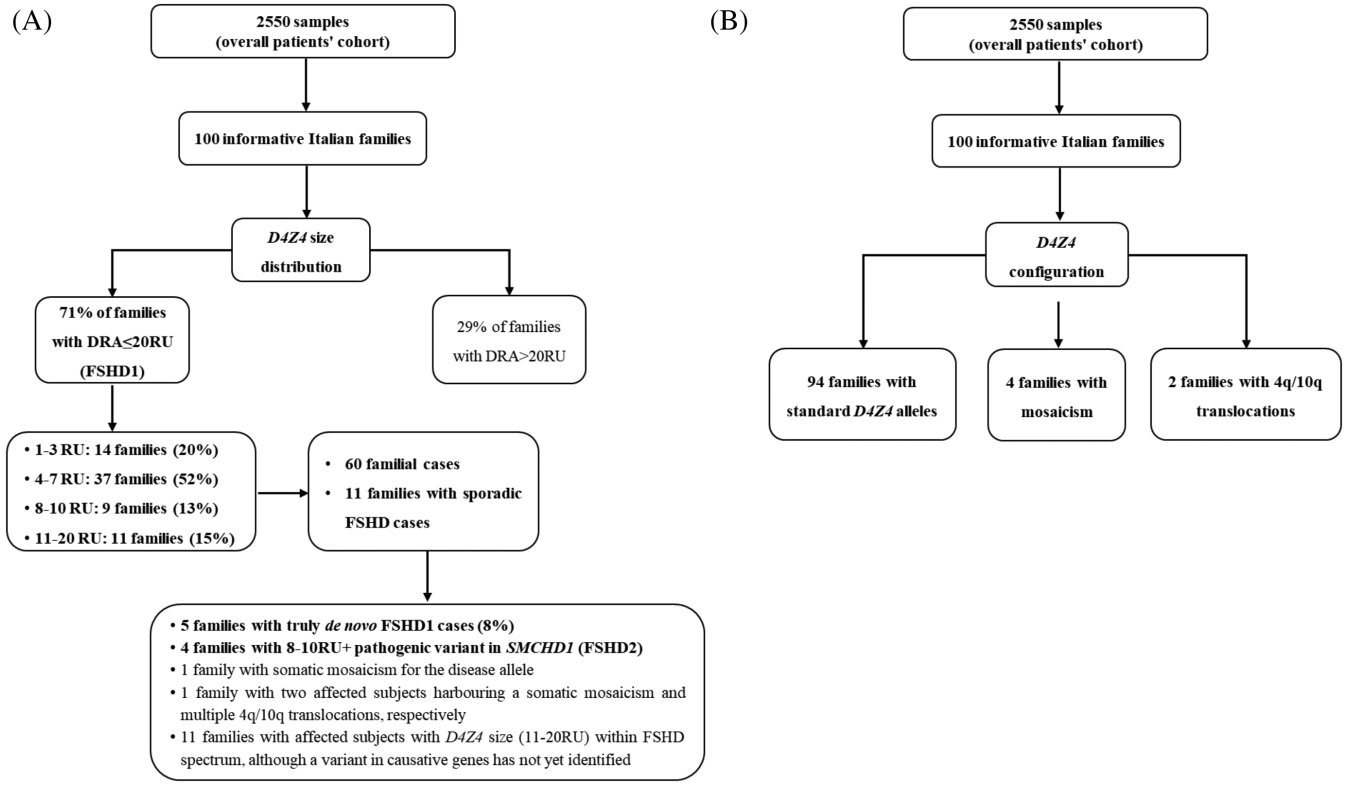

FIGURE 1 Summary results of the analysis of the 100 families, taking into account the *D4Z4* distribution (A) and configuration (B), respectively. DRA, *D4Z4* reduced alleles; RU, repeated units.

FSHD52) and 4q/10q translocations in two families (FSHD52, FSHD56). All mosaic patients were males, consistent with previous works highlighting that mosaic males are most often affected.[8]

The results obtained by the analysis of FSHD families have been summarized in Figure 1.

In the study, 29 families displayed *D4Z4* size not compatible (>20 RU) with FSHD, despite the presence of clinical signs suggestive of disease. This result may be due to the high heterogeneity and variability of disease, the presence of overlapping symptoms with other myopathies and the different experience of the medical centers referring the patients.

Overall, the present study highlighted a slightly lower frequency of de novo FSHD cases compared to previous reports. The assessment of de novo cases has important implications for genetic counseling, especially concerning the recurrence risks and the reproductive choices of families, since parents of truly de novo patients may not have a recurrence risk higher than that of the general population. However, the recurrence risk may be increased in parents carrying a germinal mosaicism or unconventional *D4Z4* rearrangements that could be responsible for apparently sporadic FSHD cases. In this regard, the gender of a parent should also be considered, since females, whether mosaic or carrying a permissive DRA, are more frequently asymptomatic than males. By contrast, affected males who have a de novo *D4Z4* contraction, have a higher chance than females for being mosaic, with consequent lower recurrence risk for their parents in any future pregnancy. Although the mechanisms leading to the sex-related differences in FSHD are not well understood, hormone factors have been proposed as potential disease modifiers.[19,20] Finally, this study further highlights the importance of performing family studies for explaining apparent sporadic, complex or de novo FSHD cases, as they can have different implications for genetic counseling, diagnosis, clinical management and procreative choices for patients and families.

Collectively, a systematic evaluation of FSHD patients and families should be recommended to describe phenotypes including typical forms of disease with different level of expression severity, incomplete/intermediate phenotypes or pauci-asymptomatic carriers. The clinical evaluation is crucial in interpreting and guiding the genetic analysis and its implication for diagnosis and familial counseling.

AUTHOR CONTRIBUTIONS

Conceptualization: Strafella C, Colantoni L, Cascella R, Giardina E. Methodology: Strafella C, Megalizzi D, Trastulli G, Proietti Piorgo E. Investigation: Strafella C, Colantoni L, Megalizzi D, Trastulli G, Proietti Piorgo E. Resources: Strafella C, Sancricca C, Ricci G, Siciliano G, Filosto M, Tasca G, Ricci E, Giardina E. Writing-Original Draft: Strafella C, Giardina E. Writing-Review and Editing: Strafella C, Colantoni L, Primiano G, Sancricca C, Ricci G, Siciliano G, Caltagirone C, Filosto M, Tasca G, Ricci E, Cascella R, Giardina E. Supervision: Strafella C, Cascella R, Giardina E. Project administration: Strafella C, Giardina E. Funding acquisition: Caltagirone C, Giardina E. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All data presented in the study are included in this published article and its supplemental files.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Santa Lucia Foundation IRCCS (CE/2022_020 approved on 01/06/2022; CE/PROG.650 approved on 01/03/2018). Written informed consent was provided by the participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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