The role of Whole-Exome Sequencing and methylation analysis in untangling complex Facioscapulo-humeral Muscular Dystrophy cases

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Background

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common forms of muscular dystrophy overall, with an estimated prevalence of 1:8.333. The pathogenic molecular mechanism is believed to reside in an aberrant expression of the *DUX4* gene resulting from the epigenetic de-repression of the *D4Z4* locus on chromosome 4q35. About 95% of subjects with clinical diagnosis of FSHD carry a *D4Z4* Reduced Allele (DRA), which is an array consisting of 11-100 tandemly Repeated Units (RUs) in the normal population and it is reduced to 1-10 RUs in FSHD patients. This is the most prevalent form of disease, and it is known as FSHD1, whereas patients carrying borderline (8-10RUs) or longer *D4Z4* sizes (usually in the 11-20RUs range) are generally affected by the FSHD2 form. In FSHD2 patients, pathogenic variants in genes (i.e. *SMCHD1*, *DNMT3B* and *LRIF1*) involved in the epigenetic control of *D4Z4* locus have been progressively identified over the last few years. Two major 4q subtelomeric variant alleles exist (4qA and 4qB), but only the 4qA (referred to as "permissive") presents a polymorphic *DUX4* polyadenylation signal enabling DUX4 expression, which is thereby responsible for a cascade of pathological processes. FSHD patients can display a wide clinical variability, even within the same family, and the availability of the genetic test has increased the diagnostic yield and led, over the years, to the identification of different phenotypes. To address the need for detailed FSHD patients' clinical characterization, the Italian Clinical Group for FSHD developed the Comprehensive Clinical Evaluation Form (CCEF) in 2016 and applied it to large cohorts of subjects. While evidence is growing about phenotypic variability and possible different disease courses, knowledge about pathophysiologic mechanisms underlying those differences is still far from being exhaustive .

Aims

The study aimed at providing a comprehensive genetic characterization encompassing the D4Z4 allele sizing, 4q haplotype identification, D4Z4 region methylation levels analysis and Whole Exome sequencing in a cohort of patients selected based on the presence of atypical phenotypic features, different phenotypes among carriers from the same kindred and disease severity unexpected from the known D4Z4 allele.

Materials and Methods

Cohort selection

Involved centers: UO Neurologia, AOUP; NeMO Center, Brescia; Osp. Le Molinette, Torino; Genomic Medicine Lab, Osp. Santa Lucia, Roma.

A total of 43 cases from 24 informative families based on:
- Atypical phenotypes (A)
- Incomplete penetrance among relatives from the same pedigree (B)
- FSHD phenotypes without compatible genetics (C)

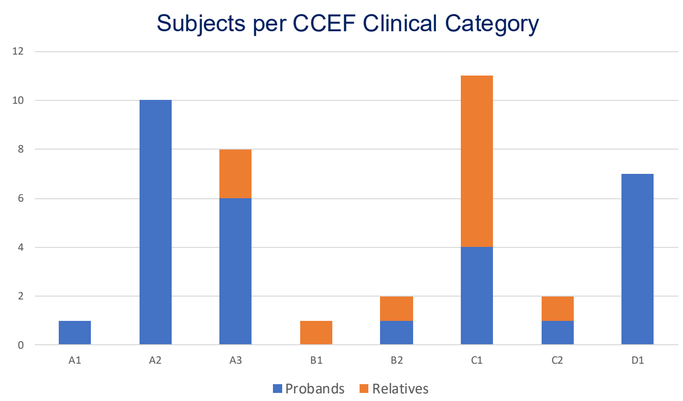
Data collection

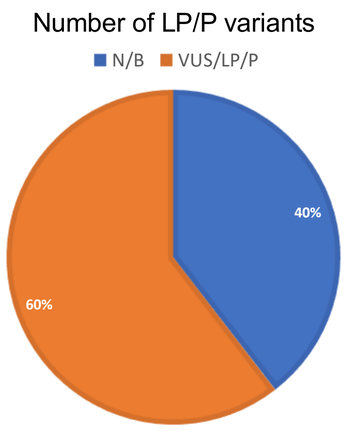
- **DRAs size**

- **D4Z4 methylation status** through Bisulfite Sequencing (BSS), Amplified Fragment Length Polymorphism (AFLP) and Machine Learning (ML)

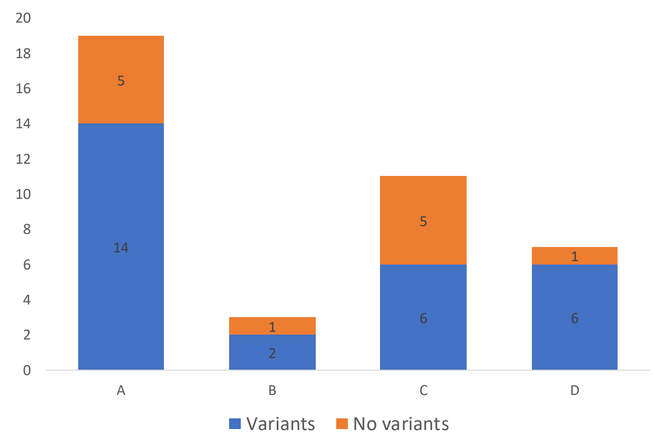
- **Whole Exome Sequencing** (Illumina® Next-Seq 550)

Results

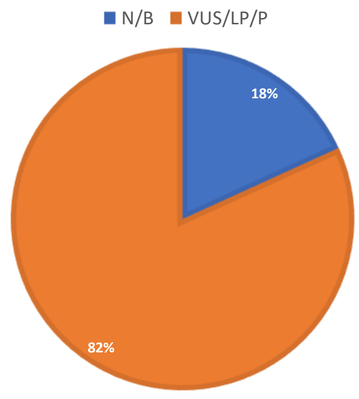


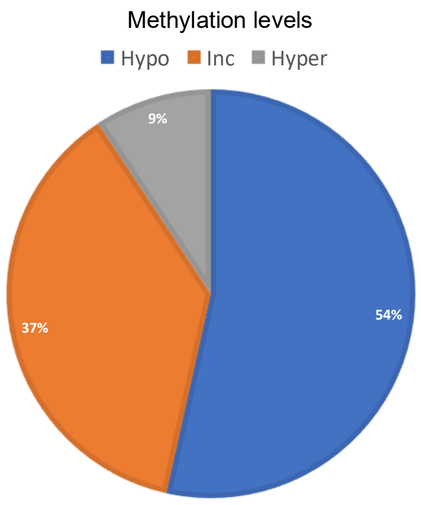


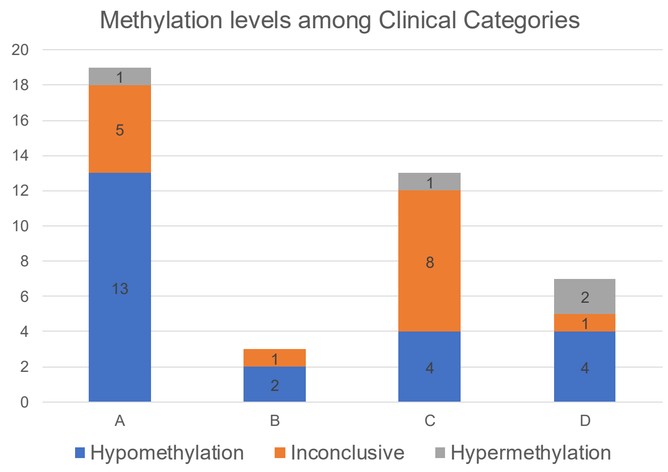
FSHD-modifying or NMD variants among Clinical Categories



Number of LP/P variants in borderline DRAs carriers

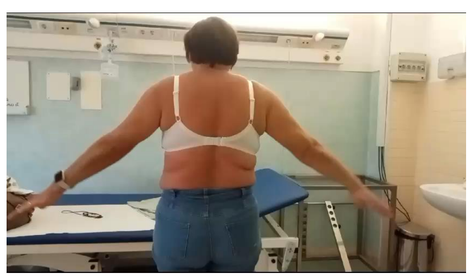


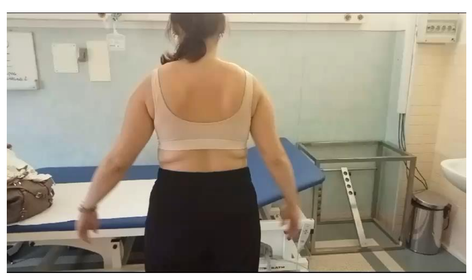




Case 1 – concordant phenotype and genetic signature

Mother (P2, 58 y.o.) and daughter (proband, P1, 20 y.o.). Scapular weakness and winged scapula in P1 starting from the age of 16, now expressing full phenotype with upper and lower facial weakness (FSHD score 8, Clinical category A2); P2 paucisymptomatic (slight winged scapula, no facial weakness, FSHD score 0, Clinical Category C1). Proband and mother have normal CK levels.
Genetic testing for FSHD1: **29 kb allele** in P1 and P2.





Methylation status:
Proband –> hypomethylated 4qA/4qA
Mother → inconclusive 4qA/4qB

WES
- P1: ***EED* (c.532A>G**, frequency 0.0003, located in the ***EZH2* interaction domain**, reported as VUS on ClinVar for autosomal dominant Cohen-Gibson Syndrome).
EZH2 has been recently described as an FSHD epigenetic regulator (*Strafella C, Whole exome sequencing highlights rare variants in CTCF, DNMT1, DNMT3A, EZH2 and SUV39H1 as associated with FSHD. Front Genet. 2023 Aug 22;14:1235589*).
This variant was not present in the mother

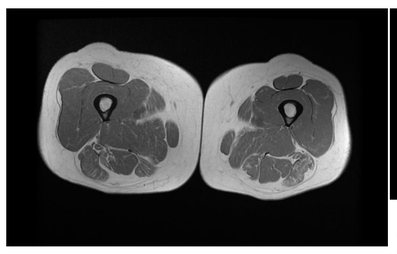
Case 2 – alternative diagnosis

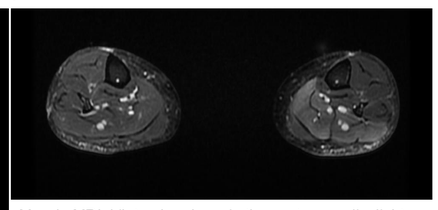
Female patient, 67 years old. At 50 mild axial weakness and later development of cardiomyopathy with Wolff-Parkinson-White syndrome and need for PM implant. CK levels were normal. A daughter with ICD implant in young age and a granddaughter deceased for sudden death. EMG study: myopathic pattern. No muscle biopsy for personal choice. Muscle CT: bilateral fatty infiltration of semimembranosus muscle. FSHD1 genetic testing: **35 kb allele, permissive haplotype**.
Methylation status: hypermethylated ; 4qA/4qB
WES analysis:

- ***TTN* likely pathogenic variant (c.72816C>A, not described, stop site gained) → also in the daughter**
- homozygous variant (c.959A>T, classified as benign and VUS on LOVD) in *AMPD1* gene

Case 3 – double trouble?

Female patient, 65 years old, no family history for neuromuscular diseases (but brothers with cardiomyopathy before the age of 50). At 58 axial weakness with camptocormia. At neurological exam bent syndrome with sloping shoulders. CK levels were high at first evaluations, then normal. Muscle biopsy: mild non specific myopathic changes. Genetic testing for FSHD1 detected a **35 kb allele** with a **permissive haplotype** and an NGS panel revealed a heterozygous ***CAPN3* variant (c.1250C>T). CCEF Cat. D1, FSHD Score 0**





Muscle MRI: bilateral oedema in the gastroncemii, slight oedema and fatty infiltration of the biceps and semimembranosus → **consistent with CAPN3**

Methylation status: hypomethylated
WES analysis:
- ***CAPN3* variant**, located in the catalytic domain, reported as pathogenic and described in ClinVar and Varsome also associated to autosomal dominant forms of LGMD

Discussion and conclusions

Our results suggest that, overall, the clinical variability may depend on the known variable penetrance of DRA (even within the same family), as well as it may rise the need for conducting further molecular analysis to exclude the presence of possible double-trouble conditions or differential diagnosis that may explain the discordance between methylation levels and clinical phenotype as shown by WES results. Starting from these premises, we believe that a proper phenotypic description taking advantage of shared tools – as the CCEF – should be the basis from which planning and interpreting molecular characterization. In the clinical trials era, profound understanding is needed to work on the right molecular targets, correctly design clinical trials and provide patients with the most suitable treatment for their disease. In the case of FSHD, this means that we should also focus on clinical features for guidance towards a phenotype-based approach to precision and personalized medicine, from diagnosis to treatment. In FSHD, clinical and genetic variability represents an obstacle for the interpretation of genotype-phenotype correlations, appropriate genetic counseling, and stratification of patients eligible for therapeutic trials. Moreover, incomplete penetrance, different pattern of muscle involvement and wide variability in disease onset and severity argue for the possible role of modifying loci or epigenetic mechanisms in FSHD. Improving the molecular diagnosis of FSHD and its consistency with clinical features is essential to better address this disease. A definite molecular and clinical characterization is essential for the development of future clinical trials that aim to be disease modifying.