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Facioscapulohumeral Muscular Dystrophy: More Complex than it Appears

G. Ricci[1,2], M. Zatz[3] and R. Tupler[*,1,4]

[1]*Department of Life Sciences, "Miogen" Laboratory, University of Modena and Reggio Emilia, Modena, Italy*

[2]*Department of Clinical and Experimental Medicine, Section of Neurology, University of Pisa, Pisa, Italy*

[3]*Human Genome Research and Stem Cell Center, Institute of Biosciences, University of São Paulo, São Paulo 05508-090, Brazil*

[4]*Program in Gene Function and Expression, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA*

Abstract: Facioscapulohumeral muscular dystrophy (FSHD) has been classified as an autosomal dominant myopathy, linked to rearrangements in an array of 3.3 kb tandemly repeated DNA elements (D4Z4) located at the 4q subtelomere (4q35). For the last 20 years, the diagnosis of FSHD has been confirmed in clinical practice by the detection of one D4Z4 allele with a reduced number (≤8) of repeats at 4q35. Although wide inter- and intra-familial clinical variability was found in subjects carrying D4Z4 alleles of reduced size, this DNA testing has been considered highly sensitive and specific. However, several exceptions to this general rule have been reported. Specifically, FSHD families with asymptomatic relatives carrying D4Z4 reduced alleles, FSHD genealogies with subjects affected with other neuromuscular disorders and FSHD affected patients carrying D4Z4 alleles of normal size have been described. In order to explain these findings, it has been proposed that the reduction of D4Z4 repeats at 4q35 could be pathogenic only in certain chromosomal backgrounds, defined as "permissive" specific haplotypes. However, our most recent studies show that the current DNA signature of FSHD is a common polymorphism and that in FSHD families the risk of developing FSHD for carriers of D4Z4 reduced alleles (DRA) depends on additional factors besides the 4q35 locus. These findings highlight the necessity to re-evaluate the significance and the predictive value of DRA, not only for research but also in clinical practice. Further clinical and genetic analysis of FSHD families will be extremely important for studies aiming at dissecting the complexity of FSHD.

Keywords: D4Z4 reduced allele, diagnostic criteria, facioscapulohumeral muscular dystrophy, genetic counseling, genetic heterogeneity, genotype-phenotype correlation, molecular test, muscle disease.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD, OMIM #158900) is the third most common form of hereditary myopathy with a prevalence of 1 in 20.000 [1]. The disease is characterized by progressive atrophy and weakness of a highly selective set of muscle groups. Before the finding of D4Z4 reduced alleles (DRA) at 4q35, which have been considered pathognomonic for disease, the diagnosis and counseling of FSHD families were entirely based on clinical evidence and family history. Over the years, DNA testing for FSHD has been considered highly sensitive and specific [2, 3]. Thus there has been a tendency to associate clinical findings and molecular data, even when the phenotype did not completely fulfill the clinical criteria of FSHD. However, the specificity and the sensitivity of DNA testing in FSHD have come into question since it was observed that 1) 3% of healthy subjects from the general population carry DRA [4-7] and 2) 20% of FSHD probands do not carry DRA [7-10]. These clinical and molecular studies show that FSHD is a more complex disorder than previously thought. Therefore the current molecular signature is insufficient to diagnose FSHD and has to be carefully re-evaluated as predictor of disease outcome. Moreover, a wide range of myopathic phenotypes have been observed in subjects carrying DRA, indicating that a careful clinical diagnosis and molecular characterization of each family should be performed to determine the significance of DRA. It is indeed possible that the lack of specificity of DRA might have led to biased interpretations of clinical observations. Additionally, genetic heterogeneity must be considered in FSHD families in which no DRA segregates. An unbiased analysis of such genealogies as well as in families with no clear autosomal dominant inheritance might allow identifying additional genes involved in FSHD pathogenesis, as it has happened in other complex genetic diseases such as Alzheimer's disease or amyotrophic lateral sclerosis [11-17]. These critical aspects must be considered both in research and in clinical practice.

*Address correspondence to this author at the Miogen Lab, Department of Life Sciences, University of Modena and Reggio Emilia, via Giuseppe Campi, 287, 41125 Modena, Italy; Tel: +39 059 2055441; Fax: +39 059 2055426; E-mail: rossella.tupler@unimore.it

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THE CLASSICAL PHENOTYPE OF FSHD

The disease was firstly reported in 1862 by Duchenne de Boulogne, who published a picture of an affected patient in his *Album de photographies pathologiques* [18]. Duchenne described the disease in his famous series of papers in Archives of General Medicine in 1869 [19], which is often cited as the earliest reference of FSHD [20]. In 1885, Landouzy and Dejerine [21] described in detail the clinical features of FSHD, thus also called "Landouzy-Dejerine form of muscular dystrophy", characterized by progressive facial, shoulder girdle and pectoral muscle weakness and atrophy, subsequent involvement of abdominal muscles with lumbar hyperlordosis and anterior leg muscles with steppage gait. Subsequently, in 1982, the thesis of Padberg provided the first modern clinical description of FSHD families. Padberg investigated a group of 107 subjects from 19 families, including 73 subjects displaying clinical signs of FSHD. These studies provided the first evidence for wide clinical variability in FSHD patients, even within the same family [22].

The clinical presentation in FSHD is characterized by initially restricted distribution of weakness starting with asymptomatic facial weakness followed by weakness of scapular fixator, humeral, truncal and lower extremity muscles. The onset at lower-extremity is often characterized by distal weakness, typically in the anterior leg compartment, presenting with footdrop. Extraocular and bulbar muscles are typically spared. Weak abdominal muscles result in a protuberant abdomen and contribute to the lumbar lordosis. Lower abdominal muscles are weaker than upper abdominal muscles, causing strikingly positive Beevor's s sign, a physical finding fairly specific for FSHD [23]. A notable distinctive feature of FSHD is that muscle weakness displays asymmetric distribution, which does not correlate with the handedness of the individual [24]. The creatine kinase (CK) level can be moderately increased or normal. Electromyography (EMG) and histological analysis reveal non-specific myopathic changes associated, in some cases, with neurogenic and/or inflammatory aspects [25, 26]. Muscle magnetic resonance imaging (MRI) can detect muscles showing normal MRI signal together with muscles showing abnormalities on T1-weighted MRI sequences, corresponding to areas of fatty fibrous replacement, or areas characterized by increased signal on T2- short tau inversion recovery (T2-STIR) sequences also in muscles not yet replaced by fat tissue, reflecting an increase in tissue water content due to muscle oedema [27]. Ancillary features, such as sensorineural deafness or retinal vasculopathy have been also reported in infantile FSHD forms, but they are not to be considered decisive criteria for FSHD diagnosis [28, 29]. FSHD has been considered a fully penetrant autosomal dominant disease with age-dependent penetrance estimated to be >95% by age 20 [30]. However, in contrast with the expected course for a classical autosomal dominant Mendelian disorder, the chronology of disease progression is unpredictable, and disease expressivity ranges from subjects with very mild muscles weakness, almost unaware of being affected, to wheelchair-dependent patients (Fig. **1**).

DEFINITION OF DIAGNOSTIC CRITERIA FOR FSHD IN PRE-MOLECULAR ERA

In 1991 an International Consortium established the clinical, laboratory and genetic criteria for FSHD diagnosis, in absence of a diagnostic DNA test. This work responded for the need of selecting families that could be included in the linkage analysis [31] towards the identification of the FSHD gene. Four main criteria were identified: (1) onset of the disease in facial or shoulder girdle muscles; sparing of the extra-ocular, pharyngeal and lingual muscles and the myocardium; (2) facial weakness in more than 50% of the affected family members; (3) autosomal dominant inheritance in familial cases; and (4) evidence of myopathic disease in EMG and muscle biopsy in at least one affected member. By contrast, (1) involvement of extra-ocular, masticatory, pharyngeal and lingual muscles; (2) regression of symptoms and signs; (3) presence of severe and diffuse contractures; (4) involvement of myocardium with presence of cardiomyopathy; (5) persistently high CK values above five times the upper limit, were considered suggestive of alternative diagnosis [31].

THE DISCOVERY OF DNA ALTERATIONS ASSOCIATED WITH FSHD

The need for an accurate pre-symptomatic test led to an active search for the identification of the FSHD gene [30]. In 1990 the FSHD locus was assigned to chromosome 4 by positional mapping performed in 10 Dutch families with autosomal dominance inheritance [32]. This chromosomal position was confirmed using additional polymorphic markers in other families [33, 34]. Subsequently, Wijmenga and coworkers [35] reported that D4S139, a Variable Number Tandem Repeat structure (VNTR) locus, was the most closely linked to FSHD. Because D4S139 represents the most telomeric 4q-specific marker, it established the location of the FSHD gene in the subtelomeric region of chromosome 4q. The assignment of the FSHD locus to region 4q35 was definitively established in 1992 by six laboratories, based on the genotyping of 504 affected patients and 559 unaffected subjects from 65 families [36]. Later, Wijmenga and coworkers [37] identified a 3.3 kb tandemly repeated sequence (D4Z4) located at the 4q subtelomeric region that could be detected by hybridization of *Eco*RI digested DNA using the p13E-11 DNA sequence as probe. This study included 11 Dutch families, 6 *de novo* cases, 29 healthy individuals. One family presenting compound heterozygosity for two D4Z4 alleles smaller than 28 kb was excluded from the study. The authors showed that in healthy individuals the majority (72%) of *Eco*RI fragments detected by the p13E-11 probe were larger than 28 kb, while in FSHD patients there was an overrepresentation of fragments smaller than 28 kb [37]. It was also shown that 5 out of

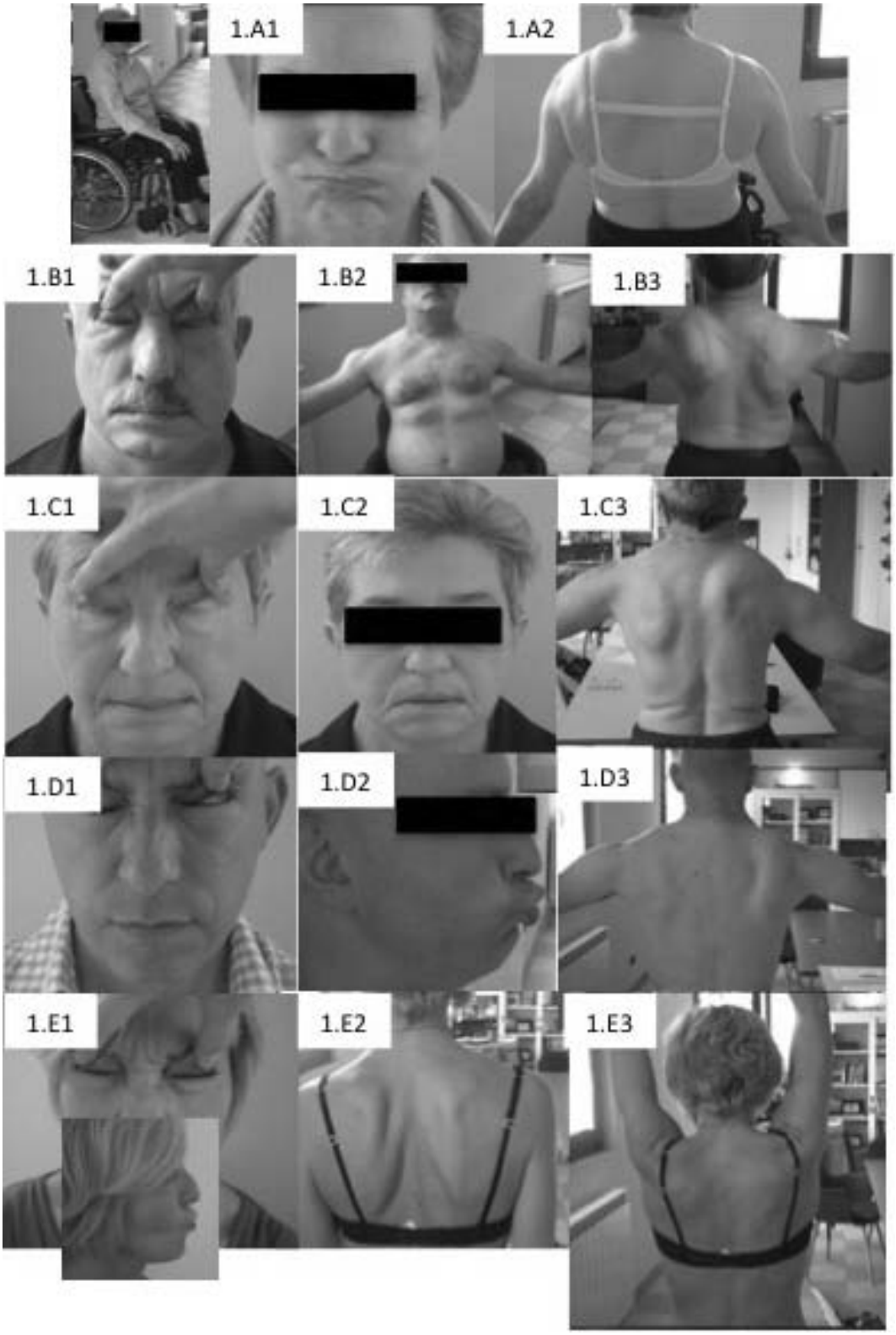

Fig. (1). Wide variability of clinical expression in a FSHD family. The proband (aged 66 years, Fig. **1A**), her brother (aged 60 yrs, Fig. **1.B1**, **1.B2**, **1.B3**), her sisters (respectively aged 65 and 52 yrs; Fig. **1.C1**, **1.C2**, **1.C3** and Fig. **1.E1**, **1.E2**, **1.E3**) and her son (aged 42 yrs, Fig. **1.D1**, **1.D2**, **1.D3**), all carrying a 23 kb 161qA DRA. The family members **1.A**, **1.B**, **1.C**, **1.D** display facial weakness (Fig. **1.A1**, **1.B1**, **1.C1**, **1.C2**, **1.D1**), limitation in raising the arms (the Fig. **1.A2**, **1.B3**, **1.C3**, **1.D3** show the maximum capacity of arms abduction), scapula winging (Fig. **1.B3**, **1.C3**, **1.D3**), fulfilling the clinical diagnostic criteria of FSHD The sister, **1.E**, aged 52 yrs, is asymptomatic. Mild scapula winging is detected at clinical examination (Fig. **1.E2**) without any motor impairment (Fig. **1.E1**, **1.E3**).

6 affected individuals with unaffected parents carried a *de novo* p13E-11 allele smaller than 28 kb. On this basis it was proposed that FSHD is caused by DNA rearrangements of p13E-11 *Eco*RI alleles. However, 8 healthy individuals presented in the study carried p13E-11 alleles smaller than 28 kb, providing an early clue that D4Z4 allele size alone would be unlikely to explain all cases of FSHD pathogenesis.

THE D4Z4 LOCUS

The probe p13E-11 detects a highly polymorphic locus with a VNTR structure constituted by a tandemly arrayed sequence of DNA repetitive elements named D4Z4 [38]. The variation in size of *Eco*RI fragments is due to variability in the number of D4Z4 repeats [39]. In normal subjects the p13E-11 *Eco*RI alleles usually range from 40 kb to approximately 300 kb (>10 D4Z4 units), whereas alleles of 35 kb or shorter (≤ 8 D4Z4 units) are present in the majority of either de novo or familial FSHD patients [40-42].

The D4Z4 repeats belong to a family of 3.3 repeats scattered within the human genome including chromosome 1 secondary constriction, and the heterochromatin of the acrocentric chromosomes [38, 43, 44]. Importantly, an almost identical D4Z4 array was located at chromosome 10q, with 98% homology between 4q35 and 10q26 regions [45]. The homology between 4q35 and 10q26 is not confined to the 3.3-kb repeats but extends both proximally (42 kb) and distally to include the telomere [46]. Notably, the size of D4Z4 alleles on chromosome 10 overlaps with those on chromosome 4.

The presence of a polymorphism on the D4Z4 copy on chromosome 10, creating a *BlnI* restriction site, has facilitated the distinction between 4q and 10q D4Z4 alleles by using *Eco*RI/*Bln*I double digestion followed by p13E-11 Southern hybridization [47] (Fig. **2**). This approach has led to the discovery that in 20-30% of the population translocated 4-type repeats reside on chromosome 10q and, viceversa, translocated 10-type repeats on chromosome 4q [48-51]. *De novo* reduced allele account for a surprisingly high percentage of FSHD patients (10%−33%) [52, 53]. This high incidence can be partly explained by the presence of parental mosaicism for 4q short alleles that has been reported in 19% of *de novo* cases [54-56]. The presence of somatic mosaicism for a rearrangement of

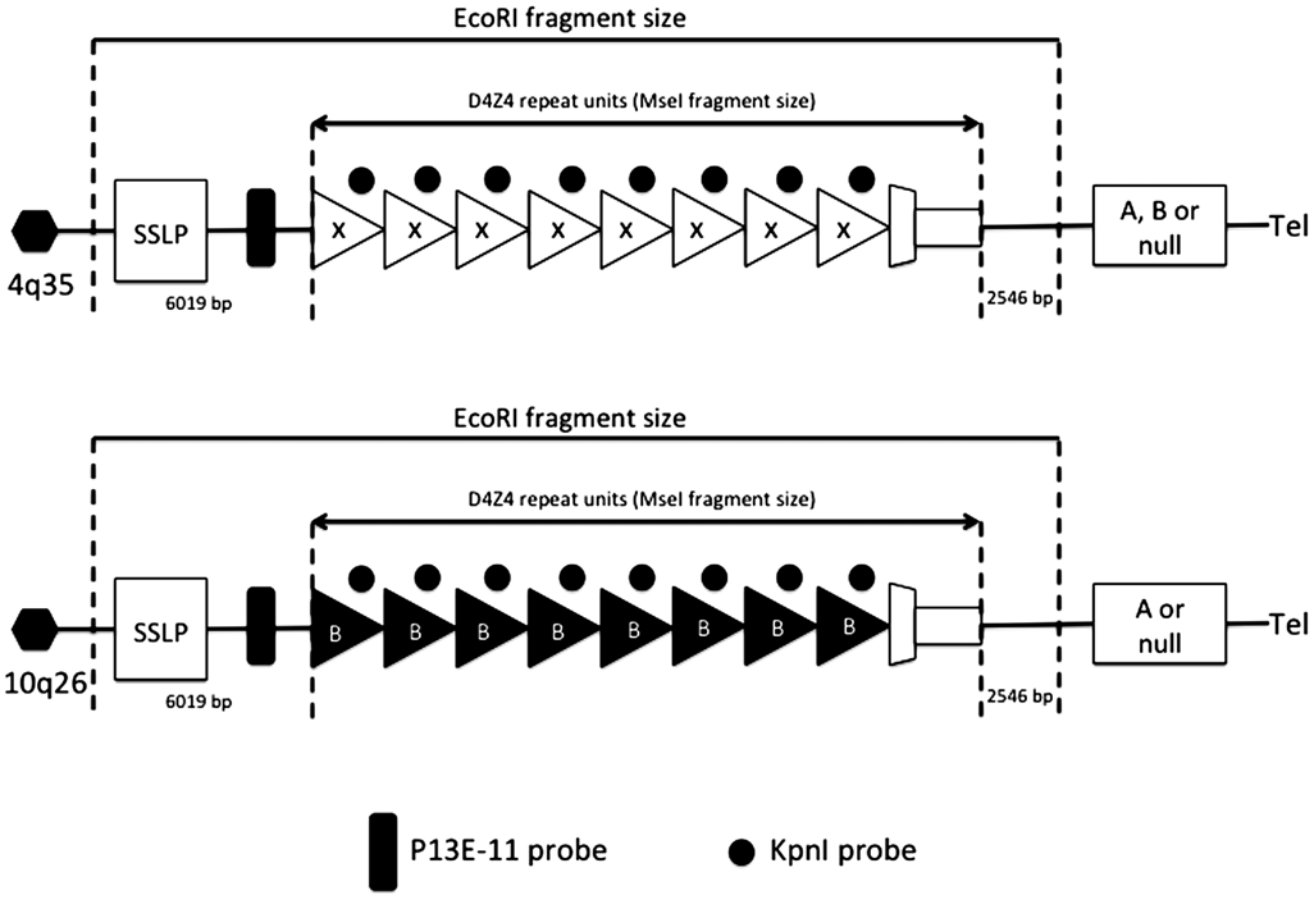

Fig. (2). Schematic representation of Polymorphisms at the 4q and 10q subtelomeres. Schematic representation of the method used to calculate D4Z4 repeat numbers from *Eco*RI-fragment sizes. Seven and eight D4Z4 repeats (31-36 kb *Eco*RI fragment size) were defined to be the upper diagnostic range for FSHD. D4Z4 repeat units on chromosomes 4 and 10 can be distinguished because all repeats on 10q contain *BlnI* restriction sites (B), while all D4Z4 repeats on 4q contain *XapI* restriction sites (X).

D4Z4 was found in as much as 3% of the general population [57]. These observations, demonstrate that the D4Z4 array is highly recombinogenic. Notwithstanding, only DRAs located at 4q have been associated with FSHD regardless of the repeat type composition.

Despite the identification of the molecular defect associated with FSHD, its pathologic effects remain largely unknown. Each D4Z4 repeat unit harbors GC-rich sequences, which are predominantly found in heterochromatic regions of the genome [38, 43] This led to the hypothesis that deletion of D4Z4 repeats might modify the chromatin organization of the 4q subtelomeric region and alter gene expression [43]. Consistently, an element within D4Z4 has been shown to behave as a silencer that provides a binding site for a transcriptional repressing complex [58]. More recent findings raise the possibility that epigenetic markings like DNA methylation [59], histone modifications [60] or chromosomal architectures [61] can be altered at the disease locus, suggesting that the chromosomal context in which the D4Z4 deletions arise can be considered crucial for clinical development of FSHD. A recent study indicates that the Polycomb group of epigenetic repressors targets D4Z4 in healthy subjects and that D4Z4 deletion is associated with reduced Polycomb silencing in FSHD patients. Cells from FSHD patients produce a chromatin-associated noncoding RNA, DBE-T, which recruits the Trithorax group protein Ash1L to the FSHD locus, driving histone H3 lysine 36 dimethylation, chromatin remodeling, and 4q35 gene transcription [62]. Collectively, these results suggest a model in which reduction of D4Z4 leads to the inappropriate transcriptional derepression of proximal chromosome 4-specific genes. Indeed, 4q35 proximal genes such as FSHD Region Gene 2 (*FRG2*), FSHD Region Gene 1 (*FRG1*), and Adenine Nucleotide Translocator 1 (*ANT1*), with high myopathic potential, were observed to be transcriptionally upregulated in FSHD muscle [58] and mice over-expressing FRG1 develop a muscular dystrophy with features of human disease [63]. Involvement of the proximal 4q35 genes added a different level of complexity on FSHD molecular mechanism other than repeats reduction, which indeed does not account for all FSHD cases. However, different studies testing this model showed controversial results. Some were in accordance [58, 64-66], while others not [67-69] preventing a consensus regarding whether protein-coding genes within 4q35 are upregulated and contribute to FSHD pathogenesis.

Another potential mechanism was suggested when detailed sequence analysis revealed that the D4Z4 repeat contains an open reading frame (ORF) encoding a double-homeobox transcription factor, *DUX4* [38, 70]. It has thus been proposed that reduction of the D4Z4 array results in the transcription of the *DUX4* [71].

Although the abundance of the *DUX4* mRNA and protein results is extremely low (approximately 1 in 1000 FSHD muscle cell nuclei were detected with an abundant amount of *DUX4* mRNA) [72], it has been observed that the *DUX4*-expressing FSHD muscle nuclei show pathologic features consistent with DUX4 induced toxicity [73]. Thus, according to the current pathogenic model of FSHD, the inefficient chromatin-mediated repression, either related to the contraction of the array or to its hypomethylation, may result in the occasional escape from repression in muscle cells with a consequently inappropriate expression of DUX4 protein [71].

In addition gene expression analyses revealed alterations in FSHD muscle that could be linked to various pathologic processes such as altered angiogenesis, susceptibility to oxidative stress and abnormal muscle differentiation [67-69, 74]. Despite all these efforts the FSHD pathophysiology and the sequence of molecular events associating a potentially cytotoxic lesion at muscle level are still elusive.

THE IDENTIFICATION OF SPECIFIC HAPLO-TYPES ASSOCIATED WITH D4Z4 REDUCED ALLELES

Since there are individuals with DRA that do not have clinical signs of FSHD, it has been proposed that additional DNA sequences flanking the D4Z4 repeat array are necessary for disease development. In 2002, a polymorphic bi-allelic segment of 10 kb distal to D4Z4 was identified [46]. Of the two allelic forms, 4A and 4B, only 4A was found to be associated with FSHD [75]. Additional sequence variations, namely Simple Sequence Length Polymorphisms (SSLP), were found proximal to the D4Z4 repeat. Together with the 4A/4B polymorphisms, these SSLPs generate at least 17 and 8 genetically distinct variants, respectively, at the chromosome 4q and chromosome 10q subtelomeres [76]. Among these many haplotypes only the common variant 4A161 and the rare variants 4A159 and 4A168 were found associated with D4Z4 reduced alleles in FSHD patients. By contrast, D4Z4 reduced alleles associated with other haplotypes were not detected in the FSHD cases. Finally, a single nucleotide polymorphism (SNP) ATTAAA was found in the pLAM1 sequence of the 4qA alleles that provides a PolyAdenylation Signal (PAS) allowing the expression of the most distal copy of the DUX4 gene [77]. Thus, it has been proposed that the combination of (1) a reduction in the number of D4Z4 elements, (2) the presence of the 4qA allele, and (3) the PAS in the pLAM1 sequence together with the (159/161/168) SSLPs represents the molecular signature that defines alleles causally related to FSHD. On this basis it has been hypothesized that this particular chromosomal setting, named 4APAS, causes FSHD through a toxic gain of function attributable to the stabilized distal DUX4 transcript [77] (Fig. **3**).

MOLECULAR BASIS OF FSHD: PATHOGENIC HYPOTHESIS

However, as explained below, data coming from different genotype-phenotype studies and clinical reports on FSHD patients and families showed that

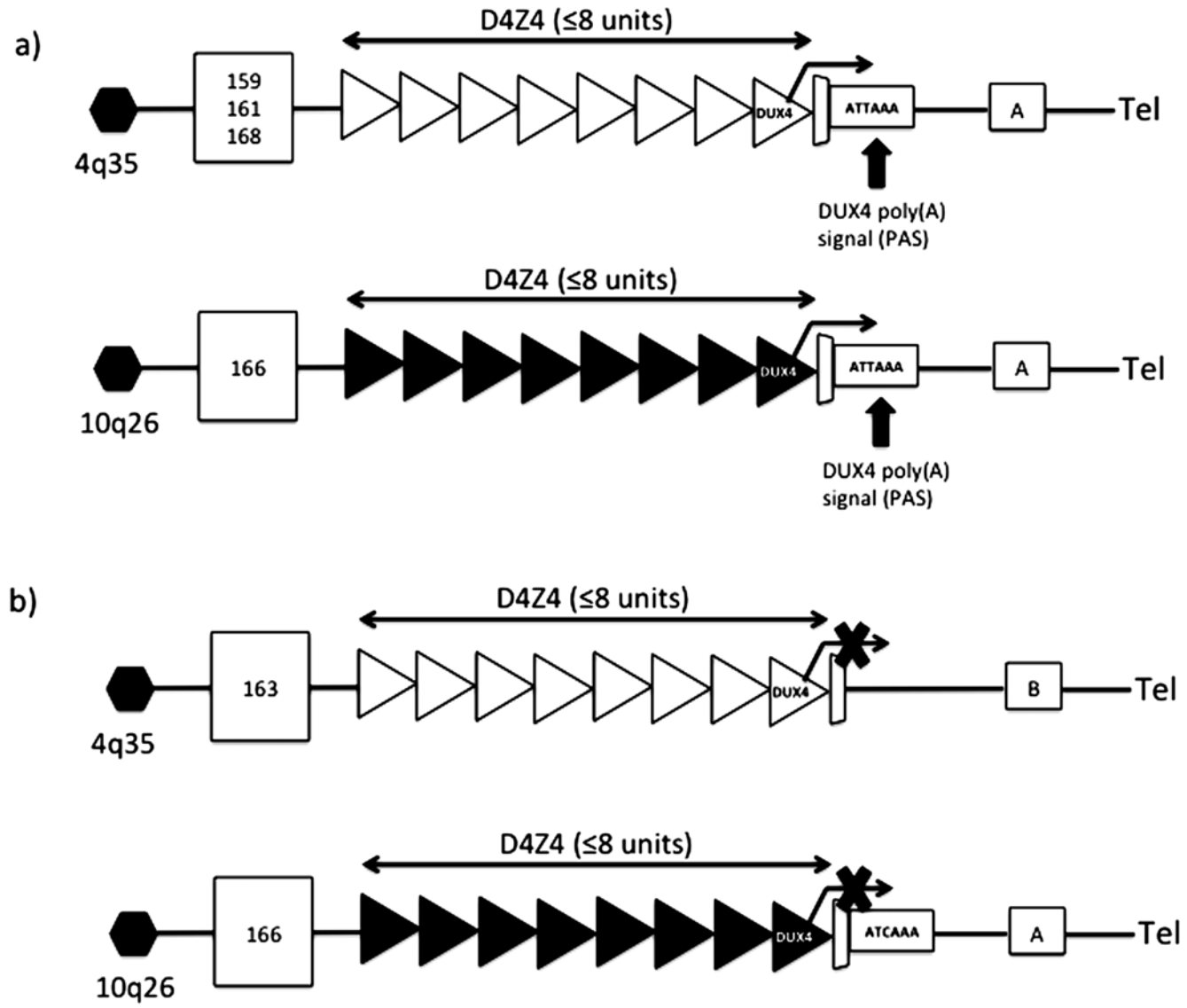

Fig. (3). Schematic representation of the current view of permissive and not-permissive haplotype. Fig. **3.A**: Permissive haplotypes. Fig. **3.B**: Non-permissive haplotype. The ATTAAA variant creates a polyadenylation signal (PAS) that stabilizes the *DUX4* transcript and has been postulated to be the critical factor causing FSHD.

exceptions that are not inconsistent with this hypothesis are frequent in human populations.

LARGE-SCALE POPULATION ANALYSIS CHALLENGES THE CURRENT CRITERIA FOR THE MOLECULAR DIAGNOSIS OF FSHD

The finding of FSHD families with compound heterozygosity [6, 78-80] suggests that in the general population DRA are more frequent than expected based on the prevalence of FSHD (1 in 20,000). This possibility was first predicted by van Overveld and coworkers [49], who found among 208 anonymous blood donors 6 subjects carrying DRA of size between 25-35 kb. This notion was definitely confirmed by the study of 801 normal control subjects from Italy and Brazil, which showed that 3% (25 of 801) of normal controls carried D4Z4 alleles of size ranging from 21 kb (4 D4Z4 units) and 35 kb (8 D4Z4 units) [7]. Remarkably 11 of them (~1.3%) carried the supposedly pathogenic 4A161PAS haplotype. The age of all these healthy carriers ranged between 40 to 78 years, an age in which FSHD is considered to be fully penetrant. Therefore it can be concluded that the current genetic signature of FSHD is a relatively common polymorphism and little predictive value can be attributed to the 4A161PAS haplotype in the absence of family history because 1.3% of healthy subjects carry this haplotype [7].

FACIOCAPULOHUMERAL MUSCULAR DYSTROPHY WITHOUT D4Z4 REPEATS CONTRACTION ON CHROMOSOME 4q35

Initially, FSHD patients were shown to exhibit one D4Z4 allele with a 8 or fewer repeats (35 kb), whereas

their non-FSHD allele shows, like control alleles, higher D4Z4 numbers (n≥ 9-100). Later, the critical number of remaining D4Z4 repeats was raised for diagnostic purposes (n=9-10) [81, 82]. At the same time it became evident that small D4Z4 alleles of 30-40 kb (6–10 D4Z4 repeats) were found also in normal controls [4-7, 83]. Thus, a remarkable overlap exists between D4Z4 alleles in controls and in FSHD patients and a definition of clear cut-off point is difficult. Furthermore it has been estimated that approximately 20% of FSHD patients carry DRA of 38 kb or larger [7-10], although these patients are clinically indistinguishable from those carrying a DRA. In 2003 Butz and coworkers conducted a systematic study including 37 unrelated myopathic patients carrying 32-41 kb D4Z4 alleles (7-10 units) and 102 healthy controls [83]. A broad myopathic spectrum with four phenotypes (typical FSHD, facial-sparing FSHD, FSHD with atypical features, non-FSHD muscle disease) was found among carriers of these alleles termed "borderline". Seven control subjects, out of 102 (6.8%), carried alleles of the same size-range. Therefore the study highlighted that in this group there is no definite D4Z4 diagnostic cut-off point separating FSHD, FSHD-like myopathies and controls, thus questioning the clinical significance of these "borderline" alleles [83].

More recently, De Greef and coworkers [84] performed a cross-sectional study on 33 patients from 27 families with D4Z4 allele with ≥ 11 repeats. These patients, termed the "FSHD2" cohort, displayed D4Z4 alleles of normal size on both chromosomes 4 alleles and appeared clinically undistinguishable from those carrying DRA. Of the 33 FSHD2 patients, 20 (61%) were males. The average age at onset was 26 years (range 0–60), which is almost 10 years later than in FSHD1. The initial symptom was scapular weakness in 61%, foot dorsiflexor weakness was reported in 27%, facial weakness in 10%, and hip girdle weakness in 3%. In contrast with the "FSHD1" cohort, in which there are significantly more males clinically and also more severely affected than females [10, 85-87] no gender difference in disease severity in FSHD2 was observed. Furthermore, there is a notable difference in the mode of inheritance between FSHD1 and FSHD2. In this study [84] pedigree analysis showed that the majority of cases (22/33) were sporadic, 11 belonged to 5 families. Two families showed autosomal dominant (parent-child pairs), two families displayed autosomal recessive inheritance (sibs pairs) and one family failed to present a clear Mendelian pattern of inheritance [84]. The major molecular feature of FSHD2 is loss of DNA methylation of the D4Z4 arrays at the 4q35 and the 10q26 [88]. Therefore it has been hypothesized that in FSHD2, in which D4Z4 alleles have normal size, there is a defect in establishing or maintaining the D4Z4 repeat chromatin structure. Consistent with this hypothesis, mutations in the *SMCHD1* (Structural maintenance of chromosomes flexible hinge domain-containing) gene have been found in FSHD2 patients [89]. This gene has been recently identified as an epigenetic modifier of chromatin structure. It has thus been proposed that lower levels of *SMCHD1* result in lower D4Z4 methylation contributing to disease onset. Therefore that lack of autosomal dominant inheritance in FSHD2 families would be explained by the presence of a mutated gene in association with D4Z4 hypomethylation.

GENOTYPE-PHENOTYPE CORRELATION STUDIES IN FSHD

Penetrance of Disease in Carriers of D4Z4 Reduced Allele

In pre-molecular era, the first observations performed on large families with clinical diagnosis of FSHD suggested an almost complete penetrance of the disease [90-92]. However, since the advent of molecular diagnosis for FSHD, subjects carrying DRA without signs of disease have been reported [4, 7, 10, 42, 80, 85-89, 93-95], challenging the notion that DRA alone can cause nearly full disease penetrance. Several such studies are summarized below.

In a previous study on 52 Brazilian families with DRA smaller than 35 kb [85], the estimated penetrance for FSHD allele was found to be 85% for patients until age 30. Furthermore, when the authors considered the sexes separately, the estimated penetrance of the FSHD allele was significantly greater for males (95%) than for females (69%). Interestingly, among 27 families with at least two clinically affected patients it was observed that in 21 families the pattern of inheritance was autosomal dominant (4 of them with incomplete penetrance). Surprisingly, in 3 pedigrees the pattern of inheritance was compatible with the presence of an autosomal recessive trait since there were at least two affected sibs born from asymptomatic parents. These observations suggested that FSHD phenotypes may result from distinct types of mutations in different families.

A study conducted on Italian families [94] reported 7 subjects, aged 20 to 69 years old, with DRA between 21 and 37 kb (4-8 units), without symptoms or signs of FSHD, who were classified as non-penetrant carriers. In this study, unaffected individuals were not observed in families with DRA smaller than 20 kb.

A retrospective analysis conducted on 85 Japanese patients with FSHD and both their parents documented parents with DRA who had no clinical symptoms, confirming an estimated low penetrance of 59% (excluding somatic mosaicism) [95].

Tonini *et al*. in 2004 [86], analyzing 238 subjects with DRA <35 kb from 106 unrelated families, observed that about 20% of individuals related to FSHD patients who carried a DRA remained asymptomatic or were minimally affected with a significantly higher proportion of females than males; asymptomatic carriers were found in about 30% of the families.

Recently, Sakellariou and coworkers [10] reported clinical and genetic analysis of 133 individuals carrying DRA (71 probands and 62 relatives) from 71 unrelated Greek families, revealing a high percentage (almost 50%) of asymptomatic relatives older than 30 years

and carrying DRA. The percentage of unaffected carriers was also lower in males than in females (29% *vs* 71%). It is also noteworthy that 16 among the 38 multiple-case families (42%) were found to have at least one symptom-free individual, with a greater proportion of asymptomatic or minimally affected gene carriers concentrating in some pedigrees, as previously observed by Tonini and coworkers [86]. A statistically significant association between the genders and the clinical manifestation of the disease was also observed: among the females the percentage of symptomatic patients was found to be 66.7% whereas among the males it was 86.6%.

The recent study performed by the Italian Clinical Network for FSHD [87] evaluated the degree of motor impairment in a large group of patients affected by facioscapulohumeral muscular dystrophy and their relatives who carry DRA. Clinical assessment was performed in 530 subjects, 163 probands and 367 relatives, from 176 unrelated families according to a standardized clinical score [96]. Overall, 32.2% of relatives did not display any muscle functional impairment. This phenotype was influenced by the degree of relation with proband, because 47.1% of second- through fifth-degree relatives were unaffected, while only 27.5% of first-degree family members did not show motor impairment. The estimated risk of developing motor impairment by age 50 for relatives carrying a DRA with 1-3 repeats or 4-8 repeats was 88.7% and 55% respectively. Male relatives had a mean score significantly higher than females (5.4 *vs* 4.0, p=0.003). No 4q haplotype was exclusively associated with the presence of disease. In 19 (13%) families in which DRA with 4-8 repeats segregate, the diagnosis of FSHD was reported only in one generation. In 5 pedigrees the pattern of inheritance was compatible with the presence of an autosomal recessive trait since there were at least two affected sibs born from asymptomatic parents.

Overall these studies on cohorts of FSHD families of different geographical origin showed that penetrance of disease is not complete, with females significantly less affected than males and in some families autosomal dominant mode of inheritance is not observed.

ATYPICAL PHENOTYPES ASSOCIATED WITH D4Z4 REDUCED ALLELES: CLINICAL SUB-TYPES OF FSHD OR MORE COMPLEX MYO-PATHIC CONDITIONS?

In the past 20 years, assessment of the D4Z4 array size as diagnostic test for FSHD has led to the identification of phenotypes that differs at various degrees from the original description of disease made by Landouzy-Dejerine [21]: two examples are shown in Figs. (**4**, **5**). This has provoked a trend towards the expansion of the clinical pattern associated with D4Z4 reduced allele. Several subtypes of FSHD with atypical clinical presentation have been described (Table **1**) [4, 83, 97-119].

For example, in 2000 van der Kooi and coworkers [102] described six sporadic cases that did not meet most of the diagnostic criteria defined in 1991 but were diagnosed as FSHD because they carried a DRA (range 26 to 38 kb) on 4q. The foot drop was the predominant clinical feature found in three patients; in three others, inability to walk on toes, shoulder pain, and pelvic limb weakness with difficulty in walking were reported, respectively. None of them had facial weakness and only one complained of shoulder weakness. Interestingly, none had a positive family history.

In the same year, Felice and coworkers [103] described 10 patients out of 14, with facial-sparing scapular myopathy associated with DRA (range 20 to 39 kb). Except for the absence of facial weakness, most patients had clinical and laboratory features otherwise consistent with FSHD. Five patients referred also a positive family history of similar weakness, although DNA analysis was not performed on other family members.

Felice and Moore in 2001 [104] also described four patients, each harboring DRA (range 25 to 34 kb), who presented with atypical phenotypes including facial-sparing scapular myopathy, limb-girdle muscular dystrophy (LGMD) distal myopathy and asymmetric brachial weakness. Only the first two patients had undergone muscle biopsies, which showed unspecific dystrophic features. None of these patients were subjected to other molecular investigations for differential diagnosis. Interestingly, the patient with LGMD phenotype and asymmetric brachial weakness did not report a positive family history for neuromuscular diseases. In this work, the authors concluded that the availability of the DNA test, considered as highly sensible and specific, allowed to establish definitively the diagnosis without the need for the more invasive and less specific muscle biopsy.

Krasnianski *et al*. [109] described three patients from a single family (father and two sons) in which a 23 kb DRA segregated. They showed signs consistent of typical FSHD associated with chronic progressive external ophthalmoplegia. The oculomotor impairment was reported as the initial manifestation of disease starting from infancy. The muscle biopsy of the father and one child demonstrated prominent myopathic changes without ragged red fibers or histopathological features of other neuromuscular diseases. The absence of singular or multiple deletions of mitochondrial DNA apparently excluded a coincidental diagnosis of Chronic Progressive External Ophthalmoplegia (CPEO) of mitochondrial origin. On the other hand, the classic FSHD distribution of the muscle weakness had been never described in patients with CPEO. The possibility of oculopharyngeal muscular dystrophy was not investigated. In the same paper [106], the authors further described other two familial cases and one sporadic case with facial-sparing FSHD syndrome associated with D4Z4 reduced allele (34 and 30 kb allele respectively).

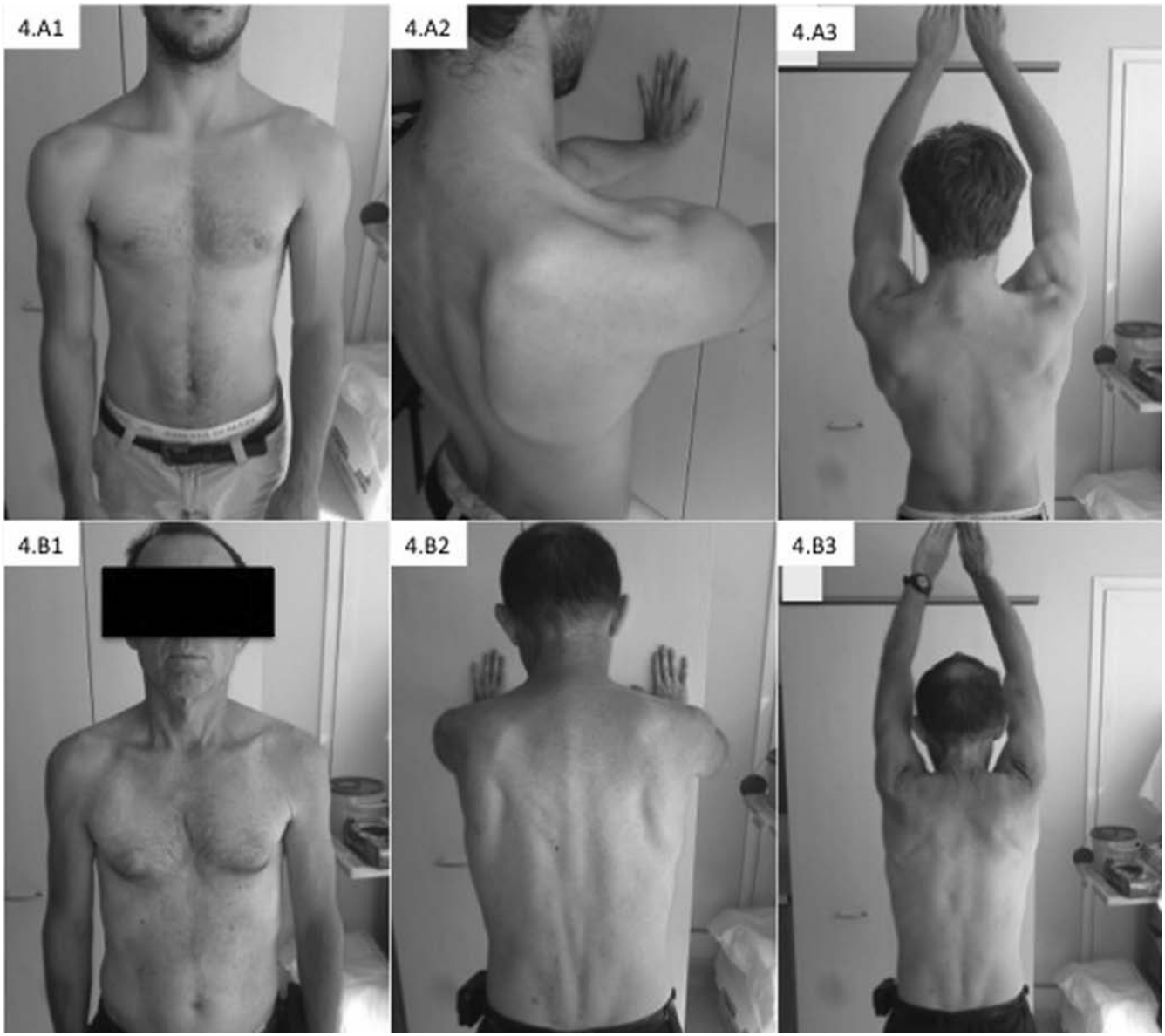

Fig. (4). A myopathic subject and his father, both carriers of a 25 kb 4qA161 DRA. The proband **4.A** (19 yrs old) complained a mild impairment of the right shoulder girdle. The neurological examination shows mild winged scapula in pushing against a wall with the hands at shoulder level and elbows straight (Fig. **4.A2**), hypotrophy of right *sovrapinatus* (Fig. **4.A2**) and *pectoralis* muscles (Fig. **4.A1**), with no limitation of arm abduction (Fig. **4.A3**). Facial weakness is not detected. The neurological examination of the father **4.B** (59 yrs old) is normal (Fig. **4.B1**, **4.B2** and **A.B3**).

Cardiac involvement, including hypertrophic cardiomyopathy, conduction defects and arrhythmia, has been reported in subjects carrying a DRA by several reports [98-101], although the European Expert Group on FSHD in 1991, that defined the Diagnostic Criteria for FSHD in pre-molecular era [31], defined that "cardiomyopathy is not part of the disease" and "when present it suggests an alternative diagnosis".

Reilich *et al*. [113] described five unrelated cases carrying DRA whose biopsies showed signs of vacuolar myopathy with rimmed vacuoles. The atypical clinical features included a form of LGMD phenotype with facial-sparing, a form of distal and proximal weakness, which was associated with dysphagia in one patient and a form of a prevalent asymmetric lower limb distal weakness. Scapular winging or facial weakness was also reported, suggesting the possibility of an overlapping FSHD syndrome. In these cases the family history was negative for neuromuscular disorders or motor impairment, although molecular analysis was not performed in other family members. Only in one family the DNA testing revealed the same DRA (size 35 kb) in the mother and two sisters of the proband affected by distal weakness; these relatives showed a mild facial involvement at clinical examination. The five muscle biopsies of the above unrelated cases showed a pattern of degenerative myopathy with rimmed vacuoles and inflammatory infiltrates. Immunohisto-

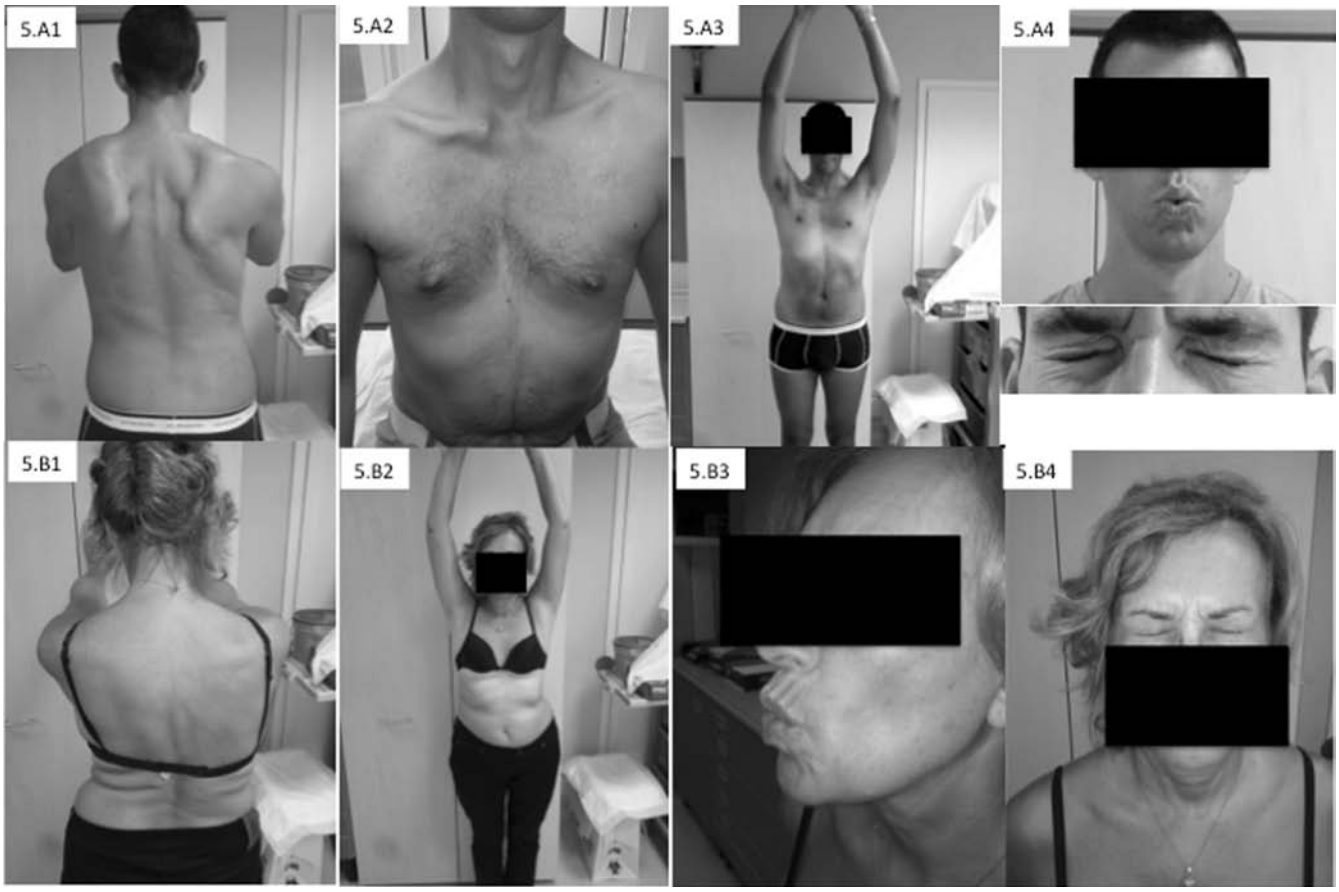

Fig. (5). A myopathic subject and his mother, both carriers of a 31 kb 4qA161 DRA. The proband **5.A** (29 yrs old) complained a mild impairment of the shoulder girdle. The neurological examination shows bilateral winged scapula (Fig. **5.A1**) and frank *pectus excavatum* (Fig. **5.A2**), with no limitation of arm abduction (Fig. **5.A3**); no evident facial weakness is detected (Fig. **5.A4**). The mother **5.B** (63 yrs old) reported congenital hip dysplasia and suffered of scoliosis since infancy (Fig. **5.B1**). The neurological examination does not show muscle impairment (Fig. **5.B1**, **5.B2**, **5.B3**).

chemistry did not detect abnormal desmin, myotilin or alphabeta-crystallin deposits, excluding the diagnosis of myofibrillar myopathies. Electron microscopy revealed autophagic vacuoles containing myelin-like material and filamentous nuclear inclusions. Interestingly, MRI imaging did not reveal the muscle lower limbs involvement typical of FSHD.

Table **1** summarizes other several atypical phenotypes associated with DRA, including the bent spine syndrome, a clinical condition characterized by a stooped posture in the standing position, which is exaggerated in walking or in exercise and disappears in the supine position, sometimes associated with a dropped head [107, 110, 115, 116]. The first reported case [107] was about a 59-years-old woman with a family history of FSHD presenting with an overlapping condition with camptocormia, scapular winging and mild facial and proximal weakness. Kottlors *et al*. [115] described the case of a 65-years-old man complaining of lower back pain and progressive bent spine syndrome, since the age of 60, carrying a 31 kb DRA. The patient recalled that his mother had a similar posture that began at age of 80. The genetic analysis performed on the available family members revealed the presence of DRA in the two daughters, who showed signs of myopathic facies. In one slight weakness of foot extensors was observed. Nonetheless, none in the family presented a typical FHSD phenotype. Jordan and coworkers [116] reported six sporadic cases carrying a DRA (range 21-34 kb) with prevalent axial weakness. All patients referred late disease onset in fourth-sixth decades. Muscle MRI imaging revealed that in all six patients the most severely affected muscles were the thoracic and lumbar spinal tract together with hamstrings.

The conclusion of some authors is that the extensive use of genetic analysis has expanded the clinical and morphological spectrum of FSHD, and many consider the detection of DRA in a patient sufficient to diagnose FSHD [3]. Interestingly, the atypical phenotypic cases are often sporadic. It may thus be supposed that in these cases the shorter D4Z4 fragment is not per se sufficient to trigger myopathy. Indeed the wide heterogeneity associated with alterations on chromosome 4q35 can suggest that other factors/pathologic conditions influence and

Table 1. Synopsis of atypical FSHD patiets and families.

Reference	Case Description	Family History
Jardine *et al*., *Neuromuscul Disord* 1994 [97]	Dominantly inherited muscular dystrophy with onset in the shoulder girdle and later progression to the lower limbs, associated with DRA of 38 kb	Familial cases
Nakagawa *et al*., *Internal Medicine* 1997 [4]	6 cases with severe limb and girdle muscular weakness (LGMD-like) with or without mild facial muscle involvement (DRA size range: 13 to 24 kb)	-5 familial cases -1 sporadic case
Laforêt *et al*., *Neurology* 1998 [98]	5 FSHD subjects with conduction defects or arrhythmia without associated cardiovascular risk factors	-3 familial cases -2 sporadic cases
Finsterer *et al*., *Cardiology* 2005 [99] **Emmrich *et al*., *Z Kardiol* 2005 [100]** **Tsuji *et al*., *Neuromuscular Disorders* 2009 [101]**	3 unrelated subjects with FSHD and cardiomyopathy	-2 familial cases -1 sporadic case
van der Kooi *et al*., *J Neurol Neurosurg Psychiatry* 2000 [102]	FSHD cases with atypical presentation, characterized by foot drop (3 cases), shoulder pain and pelvic limb weakness (3 cases). Range of DRA size: 26 to 38 kb	All sporadic cases
Felice *et al*., *Neurology* 2000 [103]	10 cases with facial sparing scapular myopathy (range of DRA size: 20 to 39 kb)	-5 familial cases -5 sporadic cases
Felice and Moore, *Muscle Nerve* 2001 [104]	1 subject with facial-sparing scapular myopathy (DRA size 25kb), 1 subject with limb-girdle muscular dystrophy (LGMD) (DRA size 34kb), 1 subject with distal myopathy (DRA size 30kb), and 1 subject with asymmetric brachial weakness (DRA size 34 kb)	-1 familial case -4 sporadic cases
Yamanaka *et al*., *Neurology* 2001 [105]	7 subjects with a severe form of FSHD associated with atrophic tongue (DRA size range: 10 to 17 kb)	-2 familial cases -3 sporadic cases
Uncini *et al*., *Neuromuscul Disord* 2002 [106]	A case with isolated monomelic atrophy of lower limb with calf muscle involvement (DRA size 26 kb)	Familial case
Umapathi *et al*., *J Neurol Neurosurg Psychiatry* 2002 [107]	A case with camptocormia, scapular winging and mild facial and proximal weakness	Familial case
Krasnianski *et al*., *Arch Neurol* 2003 [109]	3 subjects from a single family with FSHD and chronic progressive external ophthalmoplegia (DRA size 20 kb); 2 related subjects respectively with facial-sparing scapulohumeral and chronic pain/calf atrophy/mild minimal hip flexor paresis (DRA size 34 kb); a case with sporadic facial sparing scapulohumeral (DRA size 30 kb)	-5 familial cases -1 sporadic case
Butz *et al*., *J Neurol* 2003 [83]	6 subjects with facial-sparing FSHD, 4 subjects with atypical FSHD (onset and predominance in left pelvi-femoral muscle, isolated atrophy M.pect., predominant weakness of axial and pelvic girdle, one-sided atrophy of Mm. Pect., trap., suprasp.), 4 subjects with no FSHD phenotype (bilateral atrophy of Mm. tib. ant., onset in lower limbs with dysarthria and dysphagia, discrete facial paresis with highly elevated CK, improvement under cortisol, onset and predominance in pelvic muscles). DRA size range: 32 to 45 kb	-3 possibly familial cases -12 sporadic cases
Wood-Allum *et al*., *Neuropathol Appl Neurobiol* 2004 [110]	A case with clinical features of FSHD, but also kyphosis, weakness of neck flexion, and nemaline rods at muscle biopsy (DRA size 17 kb). A case with camptocormia due to weakness in the paraspinal muscles (DRA size 30 kb)	-1 familial case -1 sporadic case
Sugie *et al*., *Neurology* 2009 [111]	A case with hemiatrophy (DRA size of 20 kb)	Sporadic case
Zouvelou *et al., J Clin Neurosci* 2009 [112]	A case with persistent, asymptomatic hyperCKemia (DRA size 23 kb)	Sporadic case
Reilich *et al*., *J Neurol* 2010 [113]	5 unrelated cases with an unusual phenotype (LGMD phenotype with facial sparing, distal and proximal weakness, dysphagia, prevalent asymmetric lower limb distal weakness) and vacuolar myopathy with rimmed vacuoles	-2 possibly familial cases -3 sporadic cases
Figueroa *et al*., *J Neurol* 2010 [114]	2 siblings, one with isolated facial diplegia and the other with late onset facial and limb-girdle weakness (DRA size of 25 kb)	Familial cases
Kottlors *et al*., *Muscle Nerve* 2010 [115]	A case with lower back pain and progressive bent spine syndrome	Familial case
Jordan *et al*., *J Neurol* 2011 [116]	6 cases with bent spine syndrome (DRA size range 21 to 34 kb)	-2 familial cases -4 sporadic cases
Papadopoulos *et al*., *Muscle Nerve* 2011 [117]	A case with bent spine syndrome (DRA size 28 kb)	Familial case
Hassan *et al*., *Muscle Nerve* 2012 [118]	7 subjects with focal weakness (3 subjects with monomelic lower limb atrophy and weakness, 2 subjects with upper limb unilateral weakness or atrophy, 2 subjects with axial weakness)	Familial cases

modulate the disease expression, such as epigenetic or environmental factors, concomitant inflammatory disease. It may plausible that other genetic and/or environmental factors may participate in the onset of a myopathy that might present clinical features overlapping with FSHD. On the other hand, it must also be considered the possibility that other myopathies might have been misdiagnosed because of the random finding of a DRA in the affected subject.

SEVERAL REPORTS OF "DOUBLE TROUBLE" CONDITIONS IN FSHD FAMILIES

In 2002, Tonini and coworkers [120] reported two unrelated Brazilian families with members apparently affected by two different forms of muscular dystrophy. In the first one, the 35-years-old male proband showed LGMD with proximal weakness, elevated CK (16-fold above normal) and a myopathic muscle biopsy. Muscle protein immunohistochemical and immunoblotting analysis revealed a normal pattern for dystrophin, the four sarcoglycans, calpain, dysferlin and telethonin; DNA analysis for caveolin-3 gene was negative. Two of his sisters also complained of muscle weakness. The younger sister, aged 38 years, complained of proximal muscular weakness in upper and lower limbs, had calf hypertrophy, and a serum CK 5-fold above normal but she refused further investigations. The oldest sister, aged 51 years, showed mild clinical signs possibly consistent with FSHD, confirmed through the molecular analysis (30 kb DRA). The DRA was also found in another six relatives: four of them, aged 72, 45, 36 and 22 years, were asymptomatic and two (aged 19 and 16 years) showed only mild facial hyposthenia. Surprisingly the DRA was not detected in the affected proband. In the second family, a 57-years-old male with a typical FSHD phenotype was carrying a 17 kb DRA, which was also present in other affected relatives. However, in a 14-year-old severely affected male cousin, confined to a wheelchair since age 12, but without facial weakness, the small fragment was not found; the patient refused to undergo muscle biopsy. These families illustrate complicated situations that may occur for diagnosis and genetic counseling of neuromuscular disorders. Considering that the prevalence of hereditary neuromuscular disorders is very approximately 1/1000, we estimated that the finding of two families with an additional neuromuscular disorder was about three times higher than expected. Therefore, although the presence of different neuromuscular disorders in the same genealogy could be only a coincidence, we speculated that some epigenetic mechanisms present in particular families might turn individuals more prone to pathological mutations.

However in FSHD, more than in other neuromuscular disorders, several "double trouble" conditions patients are described. In these patients the D4Z4 reduced allele is associated with a well-known pathogenic mutation of other genes, causing complex and overlapping phenotypes as summarized in Table **2**. In particular, patients with mitochondrial myopathy/FSHD [121], Becker dystrophy/FSHD [122], Duchenne dystrophy/FSHD [123, 124], Leber's hereditary optic neuropathy/FSHD [125], LGMD1C with rippling disease/FSHD [126], myotonic dystrophy type 1/FSHD [127] were reported suggesting the possibility of a synergistic effect of those simultaneous mutations in reaching and in modulating the clinical expression.

Besides, the coexistence of facioscapulohumeral muscular dystrophy and myasthenia gravis in a same patient was also reported [128].

CONCLUSION

In the pre-molecular era, the diagnosis and counseling of FSHD families was entirely based on clinical evidence [30]. In 1992 the discovery of rearranged D4Z4 alleles generated a radical shift in the FSHD field [37]. Over the years, DNA testing of the D4Z4 locus and flanking polymorphisms has been considered highly sensitive and specific and extensively used to diagnose FSHD [3] and researchers have been trying to explain how rearrangements of repetitive elements at the 4q35 subtelomere might cause disease. Definitively, this region represents an important example of how repetitive elements can influence gene expression, and generates pathology [129]. However, several types of evidence challenge the current understanding the molecular basis for of FSHD: 1) 20% of FSHD cases do not carry alleles with reduced numbers (≤ 8) of D4Z4 repeats at 4q35 [7-10] and not all of them can be explained by DNA hypomethylation of the D4Z4 array; 2) alleles with reduced numbers (≤ 8) of D4Z4 repeats at 4q35 combined with 4A(159/161/168) PAS haplotype, have a frequency of 1.3% among healthy subjects from the general population. Thus there are millions of individuals carrying this molecular signature who do not have FSHD disease [7]; 3) the penetrance of the disease among relatives of FSHD patients is incomplete and several factors, including the genetic background [86, 87], play a role in disease outcome; 4) other genetic mechanisms should be considered to explain this large percentage of cases in which FSHD does not segregate in an autosomal dominant mode of inheritance [87] (Fig. **6**).

All these findings are not surprising if considered within the present context of human molecular genetics.

The extensive use over the past 20 years of DNA analysis for studying Mendelian disorders has revealed many disease mechanisms that are more complex than single mutations. For example, identical phenotypes may be produced by mutations in different genes [130], the same mutation can cause different phenotypes [131, 132], and distinct mutations in the same gene may result in different disorders that segregate with diverse Mendelian or even multifactorial patterns [133]. In addition, the incomplete penetrance of some mutations argues for the role of modifying loci or epigenetic mechanisms influencing the clinical expression in many Mendelian disorders. Moreover the

Table 2. Synopsis of documented genetic comorbities associated with the FSHD.

Reference	Molecular and Clinical Findings
Lecky *et al*., *Neuromuscul Disord* 1991 [123]	Duchenne dystrophy FSHD
Chuenkongkaew *et al*., *Eur J Neurol* 2005 [125]	Leber's hereditary optic neuropathy (G11778A mutation mutation in mitochondrial DNA) / FSHD (DRA size 17-27-kb)
Korngut *et al*., *Neuromuscul Disord* 2008 [124]	Duchenne dystrophy (deletion c.367_368delGT in exon 6 of the dystrophin gene) FSHD (DRA size 31 kb)
Rudnik-Schöneborn *et al*., *Neuromuscul Disord* 2008 [122]	Becker dystrophy (donor splice site mutation c.4071+1 G>T in exon 29 of the dystrophin gene) FSHD (DRA size 28 kb)
Filosto *et al*., *Neuromuscul Disord* 2008 [121]	Mitochondrial myopathy (heteroplasmic transition T12313C of the $tRNA^{Leu(CUN)}$) FSHD (DRA size 25 kb)
Ricci *et al*., *Neuromuscul Disord* 2012 [126]	LGMD1C with rippling disease (heterozygous *CAV3* T78M) FSHD (DRA size 35 kb)
Masciullo *et al*., *Neuromuscul Disord* 2013 [127]	Myotonic dystrophy type 1 (CTG expansion at the DMPK locus, about 500 repeats) FSHD (DRA size 24 kb)

possibility of conducting extensive studies in different human populations has revealed the large variability of human DNA variations blurring the distinction between a polymorphism and a detrimental mutation more subtle. Thus, establishing the value of mutational events underlying genetic diseases may be complex even in diseases with simple patterns of inheritance and well-characterized pathologic course [7, 134, 135].

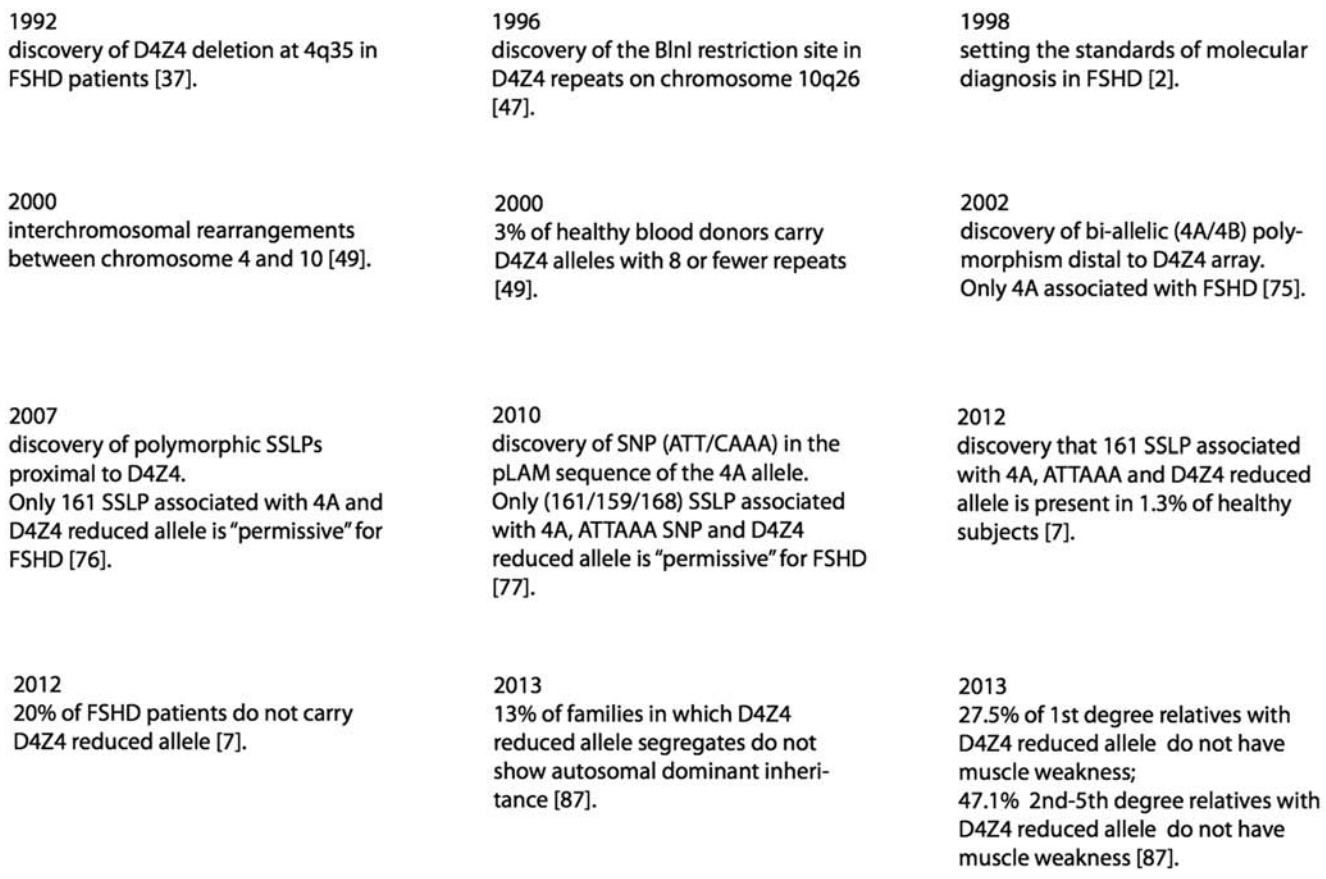

Fig. (6). Synopsis of findings in 20 years of FSHD genetic studies. The extended use of D4Z4 analysis in FSHD has challenged the original notion that FSHD is a fully-penetrant autosomal dominant disease associated with reduction in size of D4Z4 alleles.

FSHD seems to fall in this complex pattern. A wide variability in clinical spectrum together with a growing number of atypical clinical presentations associated with the FHSD genetic marker has been extensively documented. Interestingly, several cases are sporadic with no clear autosomal dominant inheritance and in some families the occurrence of affected sibs with healthy parents suggests an autosomal recessive mode of inheritance. Different explanations should be considered for atypical cases previously considered as examples of the wide clinical spectrum of FSHD as well as for non-manifesting relatives usually believed examples of non-penetrance. First, clinical heterogeneity associated with DRA may indicate involvement of other mechanisms that influence and modulate the disease expression (such as genetic, epigenetic or environmental factors), thus emphasizing the concept that the current genetic signature of FSHD alone may not be sufficient to produce clinical symptoms. Second, in families that include asymptomatic members and/or atypical phenotypes, the significance of the DRA should be carefully evaluated, and whole Exome Sequencing or Whole Genome Sequencing should be conducted in an attempt to identify new susceptibility/causative factors contributing to FSHD. To reach this goal, the precise phenotypic classification of patients and families as well as the natural history of the disease and pattern of inheritance will be crucial to create parameters to subclassify FSHD patients. This approach will lay the basis for a more precise genetic counseling of at-risk families and a better understanding of FSHD pathogenesis leading to the identification of outcomes of interests for patients and clinicians to be used in clinical trials.

ABBREVIATIONS

FSHD = Facioscapulohumeral muscular dystrophy

DRA = D4Z4 reduced allele

ORF = Open reading frame

LGMD = Limb Girdle Muscular Dystrophy

MRI = Magnetic Resonance Imaging

CPEO = Chronic progressive external ophthalmoplegia

EMG = Electromyography

CONFLICT OF INTEREST

The authors have nothing to declare.

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