Workshop report

Best practice guidelines on genetic diagnostics of Facioscapulohumeral muscular dystrophy: Workshop 9th June 2010, LUMC, Leiden, The Netherlands

Richard J.L.F. Lemmers [a,*], Suzanne O'Shea [b], George W. Padberg [c], Peter W. Lunt [d], Silvère M. van der Maarel [a]

[a] Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
[b] Bristol Genetics Laboratory, Southmead Hospital, Bristol, United Kingdom
[c] Department of Neurology, Radboud University Medical Center Nijmegen, Nijmegen, The Netherlands
[d] Clinical Genetics Department, St. Michael's Hospital, Bristol, United Kingdom

1. Introduction

During the 171st European Neuromuscular Centre international workshop Standards of care and management of facioscapulohumeral muscular dystrophy (FSHD) in January 2010 [1], it was concluded that there was a need for further discussion to better define the "gold standard" for diagnostic procedures for FSHD. With the increasing complexity of the genetics of FSHD, it is important to reach an international consensus on the molecular testing methods. To this end, a meeting was held with 39 scientists from around the world at the Leiden University Medical Center on June 9, 2010 to establish consensus Best Practice Guidelines on Genetic Diagnosis of FSHD.

2. The clinical perspective

FSHD is a myopathy with a descending order of muscle involvement. As facial weakness often goes unnoticed, the most frequent presenting symptom involves scapular fixator weakness. In the occasional event that foot extensor or pelvic girdle weakness are the first signs noticed by an FSHD patient, the doctor always finds facial or shoulder weakness as well [2]. When a physician concludes a facioscapulohumeral syndrome, the odds are in favor of FSHD and genetic testing is the preferred diagnostic choice. Some physicians include CK measurement as a check to justify the waiting period for DNA results. DNA is studied for confirmation (Table 1) if a physician considers FSHD as a high probability. If he wants to exclude non-penetrance in a sib older than 18 years, he might want genetic testing for exclusion.

3. General description of the genetics of FSHD

FSHD is an autosomal dominant disorder that in the large majority of patients (>95%; FSHD1) is caused by a contraction of the polymorphic macrosatellite repeat D4Z4 on chromosome 4q35. The D4Z4 repeat array consists of repeat units that are 3.3 kb in size and which are ordered head to tail. The size of the D4Z4 repeat array ranges in size between 11 and 100 units in control individuals. In patients with FSHD1 the D4Z4 repeat array is contracted to a size between 1 and 10 units. In general, repeat array sizes between 1 and 3 units are associated with an earlier onset and more progressive and severe phenotype than arrays with >3 units. The contraction of the D4Z4 repeat array coincides with a loss of repressive chromatin markers and reduced DNA CpG methylation levels at D4Z4 [3].

Based on sequence variations in and around the D4Z4 repeat, at least 17 genetic variants of 4q35 have been iden-

* Corresponding author. Address: Department of Human Genetics, Leiden University Medical Center (LUMC), Albinusdreef 2, Postzone S-3-P, Leiden 2333 ZA, The Netherlands. Tel.: +31 71 526 9481; fax: +31 71 526 8285.
  E-mail address: r.j.l.f.lemmers@lumc.nl (R.J.L.F. Lemmers).

Table 1
Flowchart for neurologist for confirmation or exclusion of FSHD by genetic analysis. For the confirmation of FSHD additional of muscle biopsy studies should be considered when the standard FSHD analysis is negative. In these cases other test to confirm the D4Z4 contractions are recommended (see Fig. 3).

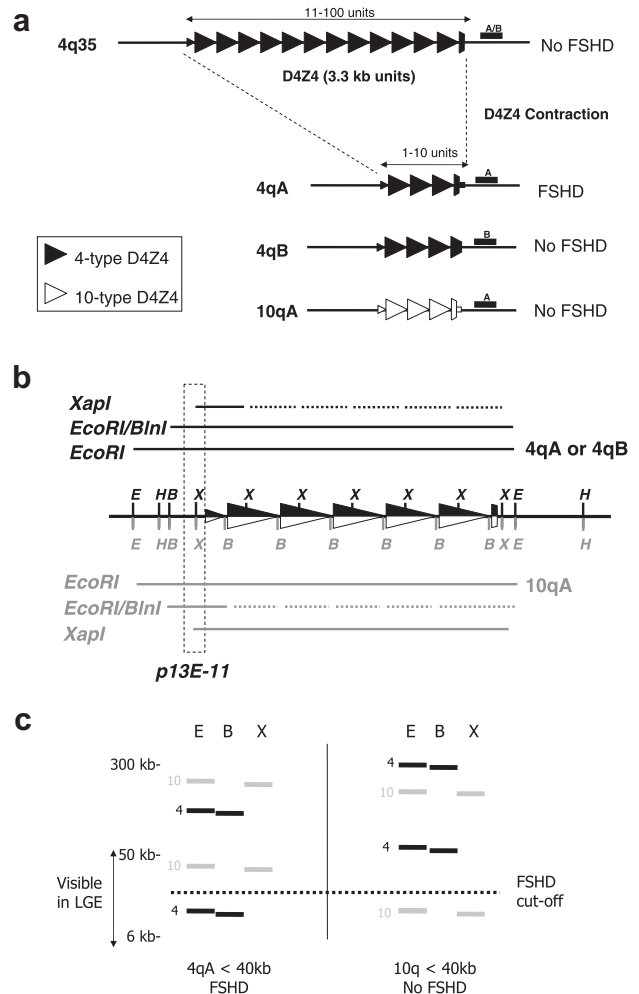| Clinical certainty	D4Z4 contraction on 4q	No D4Z4 contraction	
Confirmation FSHD	STOP	EMG Biopsy other myopathy	Rule in: p13E-11 deletion Short 4q hybrid FSHD2 (See Figure 3)
Exclusion FSHD	Rule out: Short 10q Short 4qB Short translocated 4q (See Figure 3)	STOP	

Basics for the genetic diagnosis of FSHD

Fig. 1. (a) FSHD is caused by a contraction of the D4Z4 repeat array on chromosome 4qA to a size between 1 and 10 repeat units. The D4Z4 repeat array in controls ranges between 10 and 100 units. An almost identical and equally polumorphic D4Z4 repeat array can also be found on chromosomes 4qB and 10q, but contraction on these chromosomes do not result in FSHD. (b) Southern blot discrimination between the D4Z4 homologs is based on a specific hybridization probes distal to D4Z4 (4qA/4qB) and on the restriction enzymes BlnI and Xap which specifically digest repeat units from 10q and 4q, respectively. (c) Representation of typical Southern blot analysis of D4Z4 repeats using pulsed field gel electrophoresis (PFGE). Genomic DNA is double digested with EcoRI and HindIII (E), EcoRI and BlnI (B), or digested with XapI, and, after PFGE and blotting, hybridized with probe p13E-11. The E lane typically displays four alleles, two from chromosome 4 (grey lines) and two from chromosome 10 (black lines). The B lane only reveals the D4Z4 repeats from chromosome 4, the X lane those from chromosome 10. The left genotype displays a short 4-type D4Z4 repeat and is from an FSHD patient. The right genotype has a short 10-type D4Z4 repeat and is derived from an unaffected individual.

tified that can be roughly be separated in the subgroups 4qA and 4qB (Fig. 1a) [4]. In addition, a polymorphic repeat array highly homologous to the D4Z4 repeat array can also be found on chromosome 10q (Fig. 1a) [5]. However, only D4Z4 contractions on chromosome 4qA have been reported to be associated with FSHD with the exception of a recently published case with complex D4Z4 rearrangement of chromosome 10 [6,7]. Recently it has been shown that specific sequence variations between 4qA, 10qA and 4qB chromosomes in the region distal to D4Z4 contribute to the permissiveness of the 4qA variant [7]. Thus repeat arrays <10 units on chromosomes 4qB and 10q can be frequently encountered in the normal population without apparent pathogenic consequences [8]. Yet, the occurrence of D4Z4 repeat array contractions on non-permissive chromosomes hampers the genetic analysis of FSHD.

In addition to the specificity for the genetic background to the development of FSHD also other types of rearrangements of the D4Z4 locus can complicate the diagnosis of FSHD. This includes for example gonasomal mosaicism for the D4Z4 contraction [9], or cases in which the contracted D4Z4 repeat may go unnoticed due to deletion of the diagnostic probe region [10]. Finally an epigenetic variant of FSHD has been identified (FSHD2); this variant shows similar clinical features and changes of the chromatin structure of D4Z4 in the absence of contraction [11].

4. Diagnostic techniques

Several diagnostic methods to perform the molecular diagnosis for FSHD have been developed and all are based on the determination of the length of the D4Z4 repeat array. The most common method is by Southern blotting of genomic DNA after digestion with a specific set of restriction enzymes, but some labs also use a long range PCR method [12] to size the D4Z4 repeat array. Currently, molecular combing is in development, based on fluorescence in situ hybridization (FISH) of stretched DNA molecules [13]. All three techniques use genomic DNA isolated from peripheral blood lymphocytes (PBLs), chorionic

villus sampling or cultured amniotic fluid cells, but the DNA isolation method can be different.

For the isolation of PBL from peripheral blood, the initial erythrocyte lysis protocol is comparable between the different techniques, but the handling of the white blood cell (WBC) pellets can be different. Liquid DNA can be isolated from the WBC pellet either manually [14] or in an

automatic system, which can be used for Southern blotting and long range PCR. However, the mechanical stress during the preparation of liquid DNA generally leaves genomic DNA fragments <200 kb and further shearing during the handling of the DNA might result in even smaller fragments. Therefore liquid DNA is generally not suitable to size D4Z4 repeat arrays >50 kb and as a consequence not all D4Z4 arrays from 4q and 10q in an individual will be visualized. Furthermore, the identification of all D4Z4 fragments in a single individual may assist in the identification of complex D4Z4 rearrangements like gonasomal mosaicism and D4S104F1 deletions as will be explained in the next paragraph. Ideally the analysis of the D4Z4 repeat arrays is performed on agarose embedded DNA plugs in which high molecular weight DNA can be preserved enabling the identification of all four D4Z4 repeats by Southern blotting (http://www.urmc.rochester.edu/fields-center/). These agarose plugs are also required for the FSHD diagnosis by molecular combing.

5. Southern blotting

The most common method to perform the molecular diagnosis for FSHD is by Southern blotting. For this method high molecular weight genomic DNA in plugs or dissolved in Tris buffer is digested with restriction enzymes (in general with EcoRI or with EcoRI and HindIII) that cut at both ends of D4Z4 repeat array, thereby releasing complete D4Z4 repeat arrays with little flanking sequences. For this reaction approximately 5 μg of liquid genomic DNA is required or alternatively 500,000 cells (approximately 3.5 μg DNA). Subsequently, size separation of the digested DNA is performed by linear gel electrophoresis (LGE) or by pulsed field gel electrophoresis (PFGE). LGE allows the sizing of DNA fragments between 3 and 50 kb which enables the identification of FSHD-sized fragments. However, PFGE together with agarose embedded DNA plugs allows separation of fragments up to hundreds of kilobases and therefore enables the identification of all four alleles (chromosomes 4 and 10) [15].

After separation the DNA is transferred to a Nylon membrane by Southern blotting and then the D4Z4 repeat arrays are visualized using probe p13E-11. Probe p13E-11 recognizes the region immediately proximal to D4Z4 contained within the EcoRI fragment and is a suitable probe for most cases. Southern blot hybridizations are often performed with radioactive labeled probes using the isotope phosphorus 32 (32P) with has a half-life of 14 days. However, all the major diagnostic probes currently available can also be labeled using non-radioactive techniques which do not need a special designated area or laboratory for performing the analysis and is much more stable (up to 1 year) than radioactive probes making it more suitable for smaller diagnostic centers [16].

The detection of chromosome 4-specific D4Z4 repeat arrays is complicated by the presence of an equally polymorphic D4Z-like repeat array on chromosome 10q. In general, D4Z4 sequences on chromosome 4q and 10q are about 99% identical. To discriminate between permissive 4q and non-permissive 10q chromosomes the Southern blot method takes advantage of consistent sequence variations between both repeat arrays which create different restriction enzyme recognition sites between 4q and 10q. In two separate reactions a discriminative digestion for chromosome 4 and 10 type D4Z4 repeat arrays is performed. Double digestion with restriction enzymes EcoRI and BlnI leaves chromosome 4-type D4Z4 units undigested while fragmenting chromosome 10-type units [17]. The restriction enzyme XapI does the opposite [18]. Fig. 1b illustrates how these restriction enzymes enable the discrimination between contracted D4Z4 repeat arrays from chromosomes 4 and 10. Consequently, the Southern blot-based diagnostic method for FSHD is a very informative method that enables the accurate sizing and chromosomal specificity of the D4Z4 fragments (Fig. 1c). On the other hand this method is very laborious and it requires large amounts (at least 15 μg) of high molecular weight genomic DNA. Furthermore, the whole procedure takes more then a week from peripheral blood withdrawal until the D4Z4 fragments are visualized by Southern blotting, hybridization and image exposure. The use of non-radioactive labeling can dramatically reduce the time that analysis takes due to short exposure time. In addition, the recently developed molecular combing technology may also provide a less labor-intensive alternative [13]. Finally, in a substantial number of cases complex D4Z4 repeat arrays can cause problems in interpretation or even diagnostic errors.

6. Identification of complex D4Z4 rearrangements by Southern blotting: complicating D4Z4 rearrangements

The ancestral D4Z4 repeat arrays on chromosome 4q (D4Z4 units are XapI sensitive and BlnI resistant) and its homologue on chromosome 10q (D4Z4 units are BlnI sensitive and XapI resistant) have been involved in several complex rearrangements, which resulted in hybrid repeat arrays consisting of 4q- and 10q-like repeat units on both chromosomes. About 6% of the European chromosomes 4 (12% of the individuals) carry hybrid repeat arrays and repeat contractions in hybrid D4Z4 arrays below 11 units have been shown to be pathogenic (Fig. 2a). After this duplication, the hybrid D4Z4 repeat array on chromosome 10q likely homogenized into an array with only 10-type D4Z4 units. In addition homogeneous 4 type repeat arrays have been translocated to chromosome 10q. About 8% of the European chromosome 10 belong to this ancient translocation group (16% of the European individuals) [4]. Like the common chromosome 10q, D4Z4 contractions on these 10q translocation chromosomes are non-pathogenic (Fig. 2b).

As shown in Fig. 2a and b these complicated situations can be identified using PFGE and EcoRI/BlnI and XapI digestions. In total these remnants of D4Z4 evolution give rise to complex D4Z4 profiles in almost 30% of the

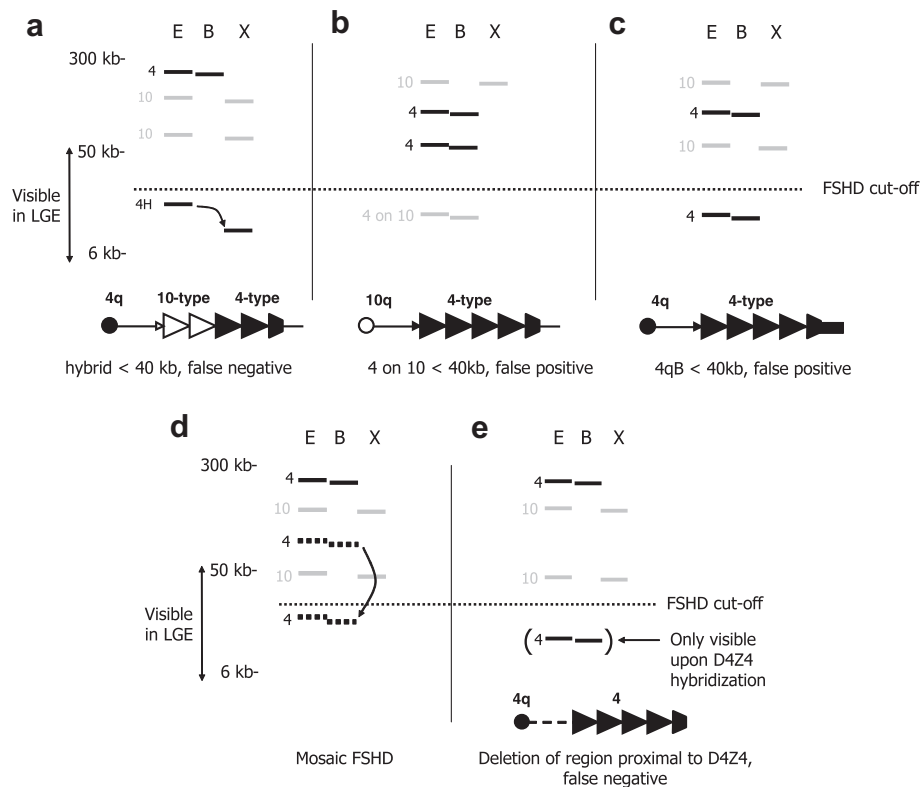## Most prevalent complex situations in the genetic diagnosis of FSHD

Fig. 2. (a) Representation of a Southern blot result of an FSHD patient carrying a short hybrid repeat array on chromosome 4, which consist of both 4-type and 10-type D4Z4 units. The first few units of this hybrid array are 10-type and as a consequence the fragment is not visible in lane B (*Eco*RI and *Bln*I double digestion). However, the *Xap*I digestion does not show a typical 10q pattern as the corresponding fragment is not visible at the expected size in lane X, like the two larger 10q fragments in the same individual (grey fragments). This complex genotype can be elucidated by the *Xap*I digestion. Normal-sized hybrid repeat arrays can be found in about 10% of the control population and therefore the prevalence of a short hybrid FSHD repeat array might also be about 10%. (b) Southern blot representing a control individual carrying a short 4-type D4Z4 repeat on chromosome 10. Typical these translocated repeat array on chromosome 10 consist of a homogeneous 4-type (*Bln*I-resistant, *Xap*I-sensitive) repeat array and is difficult to distinguish from standard chromosome 4 repeat arrays. However, contractions of these repeats do not cause FSHD. Normal-sized hybrid repeat arrays can be found in about 10% of the control population and in less than 1% of these cases the translocated repeat array is shorter than 40 kb. On a PFGE gel the presence of a translocated repeat array is clearly visible by the appearance of three 4-type fragments. Translocated repeats on 10q can end with the distal A or B variation. By performing a SSLP analysis this genotype can be recognized as translocated 4A-type repeats on chromosome 10 have unique SSLP fragments of 176 or 180 bp. Thus this complex genotype can be elucidated by PFGE and SSLP analysis.

European and Asian individuals [19]. And these cases ought to attain more attention in the assignment of the repeats to the appropriate chromosome by Southern blotting because the discriminating restriction enzymes typically used in the diagnosis are not informative. A recent population study shows that about 40% of the African individuals carry a hybrid repeat arrays on chromosome 4q [4].

Based on a large polymorphism involving a beta-satellite repeat immediately distal to the D4Z4 repeat, two variants of chromosome 4q have been detected; 4qA and 4qB, and a study in several families shows that contracted D4Z4 repeat arrays on 4qB chromosomes do not cause FSHD [8]. As non-pathogenic short D4Z4 repeat arrays on 4qB chromosomes can be detected in about 1% of the European controls they might hamper the genetic analysis for FSHD in some cases (Fig. 2c). The recognition of 4qA- and 4qB-type chromosomes requires the use of specific probes (probe A and B) for which the chromosomal DNA has

to be digested with another restriction enzyme (*Hin*dIII) [6]. Subsequent steps are similar as for the D4Z4-sizing experiment with p13E-11.

About 10–30% of FSHD patients are diagnosed with a new D4Z4 repeat contraction event. These new mutations can occur either in the germ-line or by a postzygotic rearrangement resulting in gonasomal mosaicism [20]. As shown in Fig. 2d, mosaicism for the D4Z4 contraction is unmistakable detected when performing PFGE on DNA agarose plugs by the presence of an additional D4Z4 fragment. Three fragments show a normal intensity (are present in all cells), while the two more faint bands represent the repeat array size in the two mosaic cell populations. Previous studies have shown that, while the contraction can be identified by LGE, in virtually all cases the mosaic nature of the contraction is not recognized by this technique [9]. Even if liquid DNA is used in a PFGE setting the mosaicism is often not recognized as not all D4Z4 alleles are visualized due to shearing of the repeat arrays larger than 100 kb.

It has been shown that the ratio of the mosaic cells in blood is often similar to that in muscle and probably also to that in germ line tissue [21]. Therefore, it is important that the mosaic nature of the contraction is recognized as this might have important clinical consequences and consequences for genetic counseling. Firstly, the disease in a mosaic individual is often much milder as only part of the muscle cells carry the mutation and consequently the offspring that inherits the genetic lesion is generally more severely affected than the mosaic parent. Secondly, the risk of transmitting the disease is smaller in mosaic FSHD patients as their germ-line is (likely) also mosaic [9].

In some patients the short D4Z4 repeat array remains invisible as the genetic lesion is not restricted to the D4Z4 repeat, but extends proximally and can include the D4F104S1 region that is recognized by probe p13E-11. Similar to D4Z4 mosaicism, genotypes that carry a p13E-11 deletion on one of their chromosomes can be easily detected by using PFGE on high quality DNA because in these cases the genotype only reveals three D4Z4 associated repeat arrays (Fig. 2e). Using LGE, these situations can not be distinguished from a control genotype and are often misinterpreted. Previously, these deletions were estimated to occur in approximately 3% of all the FSHD patients [10]. These proximally extended deletions can be identified by re-probing of the membrane by stringent hybridization conditions with probe D4Z4, which will visualize the missed D4Z4 fragment, or probes 4qA and 4qB [22].

Recently, an epigenetic variant of FSHD has been identified (FSHD2) that is not associated with repeat contractions [11]. This variant is clinically indistinguishable from contraction-dependent FSHD1 and can explain about 3% of the FSHD patients [23]. FSHD2 requires a permissive 4qA chromosome like FSHD1, but the loss of repressive chromatin marks and CpG hypomethylation at D4Z4 is more profound in this variant and can also be found on the D4Z4 arrays from the homologous chromosome 4q and both chromosomes 10q. Unfortunately, detection of FSHD2 is still in the research phase and is not carried out in a diagnostic setting.

7. Other diagnostic methods

As shown in the previous paragraphs, the standard Southern blotting diagnostic methods enable the identification of FSHD1 in about 95% of the cases. Some complicating genetic situations might result in false positive or false negative testing and therefore additional analysis Southern blot-based methods have been developed that increased the sensitivity and specificity. However, these methods are expensive, labor intensive and it requires large amounts of high molecular weight DNA. As currently the diagnostic tests for most diseases are optimized for high throughput sample handling there is clearly a need for an improved method to perform the molecular diagnosis for FSHD1. Therefore other methods have been introduced, which will be discussed in the following paragraphs.

7.1. Long range PCR

D4Z4 sizing can be performed by long range (LR-) PCR. LR-PCR uses specific primers that are designed proximally and distally to the D4Z4 repeat array. The PCR program takes about 13 h and the size of the D4Z4 array is visualized directly after LGE. This method enables repeat array sizing in as little as 400 ng of genomic DNA and ideally can be performed within one day [12]. However, the method does not allow the identification of repeat arrays that are >6 repeat units. In Japan the pathogenic D4Z4 repeat in patients ranges between 1 and 7 units and therefore LR-PCR allows the detection of 95% of the cases. However, in European FSHD patients the pathogenic D4Z4 repeat ranges between 1 and 10 units. Repeat array sizes between 6 and 10 units are common in most European familial cases. Therefore, the LR-PCR method can not be advised for the identification of FSHD in most European FSHD families. Another concern for this method is that in case of a negative PCR result it is unclear if either the DNA available was unsuitable for PCR amplification due to protein and salt impurities or due to low DNA quality or that the individual does not carry a contracted D4Z4 repeat array.

7.2. Molecular combing

Currently, a method based on Molecular Combing (MC) is in development that may provide a good alternative for the molecular diagnosis of FSHD [13]. By this method high quality genomic DNA from an agarose plug is stretched on a cover slip after which the D4Z4 fragments are visualized and sized with fluorescence labeled probes using fluorescence microscopy. Specific probes have been designed to recognize the region proximal to D4Z4 in a specific pattern to discriminate between chromosomes 4 and 10. In addition, probes with different fluorescence labels have been developed that recognize the p13E-11 region, D4Z4 and that discriminate between the distal A and B in the region distal to D4Z4. As the chromosome 4 and 10 discrimination for this technique is based on a large region proximal to D4Z4 rather than on partially specific restriction enzymes within D4Z4, this method overcomes many of the previously mentioned complicating factors including hybrid arrays, translocated repeat arrays and proximally extended deletions. MC for FSHD is not yet widely operational and requires dedicated personnel but might prove to be a promising new development in the diagnostic toolkit for FSHD if the D4Z4 repeat array sizing proofs to be accurate and when the microscopic analysis becomes completely automated.

7.3. Marker analysis

Another method that might assist in the molecular diagnosis for FSHD is based on DNA markers proximal to D4Z4. By using this method it is not possible to determine

the size of the D4Z4 repeat array, but it enables to study the segregation of a pathogenic chromosome after preceding detailed chromosomal analysis including D4Z4 sizing of other family members. This method has mainly been setup as a rapid screening to determine the chromosome 4 haplotype, for prenatal diagnosis and for preimplantation genetic diagnosis (PGD). Until now, several polymorphic markers proximal to D4Z4 at chromosome 4q35 have been tested for PGD but most of them are localized at a large distance from the D4Z4 repeat (0.55–1.88 Mb). In a multiplex PCR-based approach several markers were combined and technically fulfill guidelines for single-cell analysis. However, a relatively high recombination risk was shown for these large distance markers which hamper its application to PGD, as well as the absence of suitable markers distal to the repeat [24]. More recently, polymorphic DNA markers have been described that are only a few kb proximal to the D4Z4 repeat (SSLP and p13E-11 region) [4,25]. The SSLP marker is low polymorphic and is in linkage disequilibrium with the sequence variation in p13E-11. It has been shown that pathogenic contractions are associated with the most common chromosome 4qA variant 4A161 and the rare variants 4A159, 4A168 and hybrid 4qA chromosomes. Both markers have been tested for there utility as a predictive marker for segregation studies of the pathogenic FSHD chromosome. The most recent study suggests that the SNPs in the p13E-11 are informative and can be used in most cases. These results are based on the detection of the sequence variations that are associated with the 4A161 chromosome [26]. However, permissive rare variants of chromosome 4qA will be missed in the analysis as they are associated with non-4A161 variations. Furthermore, for both markers the procedure recognizes both chromosomes 4 and 10 and one non-permissive chromosome 10 variant is also associated with the 4A161 SNPs. In addition, the 4A161 chromosome is the most common 4qA variant and more than 50% of the European population is carrier of this chromosome. Therefore, the specificity of the test in a familial segregation study is low as often one of the parents is carrier of a 4A161 chromosome with a normal-sized D4Z4 repeat array. On the other hand the SSLP analysis can be useful for preventing false positive testing in case of short translocated 4qA repeat on chromosome 10q (SSLP analysis will identify a 176 or a 180 peak corresponding to a 10A176 or a 10A180T chromosome, respectively) (Figs. 2b and 3).

8. Types of Laboratory referral and their analysis

In diagnostic testing for FSHD, particularly if based on p13E-11 LGE analysis alone, there is potential for a false negative or a false positive result. A false negative result would occur where the true FSHD patient has a proximal-extending D4Z4 deletion which also deletes the p13E-11 probe region (D4F104S1 locus); or where the patient has a shortened but hybrid D4Z4 array at 4q35 containing 10-type repeats. There are also patients with FSHD2 who are clinically indistinguishable from FSHD1 but who do not have a D4Z4 contraction at 4q35, and whose diagnosis would currently rest on their clinical presentation.

A false positive result would occur where a patient who does not have FSHD, has a shortened D4Z4 fragment, either as the chromosome 10q26 homologous repeat array, or at 4q35 but linked with a non-permissive 4qB or SSLP marker haplotype.

In order to minimize false negatives and false positives, and to appropriately target further testing, all participants at the Best Practice Meeting agreed that it would be very helpful to know at the time of referral of DNA to the lab, what the clinician's expectation would be for the likelihood of the patient having true FSHD. This could simply be given by the referring clinician indicating whether the clinical diagnostic suggestion of FSHD is Definite, Probable, or Uncertain; or whether the sample is from a clinically asymptomatic family member of a previously-diagnosed case. Information on whether FSHD has been diagnosed previously in another family member should in all cases be included with the test request. From this short clinical description of a patient, and together with an estimate of the 'clinical (FSHD) diagnostic likelihood', the geneticist can decide whether to perform additional genetic analysis, or to forward on the sample to a more specialist lab for further DNA tests.

In general, if a diagnosis of FSHD is expected, and p13E-11 testing shows a typical *Eco*RI/*Bln*I shortened fragment, further testing to exclude false positives would not usually be necessary. If the clinical diagnosis is much less certain, and not as a known family predictive situation, further testing would often be recommended. In cases where FSHD is much less certain (i.e. one of a number of possible diagnoses) the absence of a typical shortened fragment would usually be taken as excluding FSHD, without further testing. It is in cases where the clinical diagnosis is definite or probable, that further tests should be performed if the initial *Eco*RI/*Bln*I test (or equivalent) is negative.

9. Experience and techniques of different diagnostic labs

There were 19 laboratories who offer molecular testing for FSHD on a service basis who provided data and were represented at the meeting. The activity for each lab varies with five labs testing 20–50 samples per year, five testing 80–150, and eight testing 150–400 per year. The electrophoretic method used is LGE alone in 10/19 labs (53%), PFGE as the primary method in 6/19 (31%), and both techniques in 3/19 (16%), including long rang PCR in one of these. Interestingly, mosaic FSHD cases were as expected mainly recognized by labs performing PFGE analysis on high quality plug DNA. In routine service testing (in 16 labs) the mean of the percentages of positive results on the index case is: 50% (range 27–95%).

For diagnostic technique, all labs commenced with p13E-11 DNA probe, and almost all (84%) run at least

two separate tracks (*Eco*RI, or *Eco*RI/*Hin*dIII and *Eco*RI/*Bln*I) on all samples. 3/19 (16%) labs perform *Eco*RI/*Bln*I together in a single track. This latter approach may reduce cost but will inevitably result in either false negative (Fig. 2a) or false positive results due to the presence of FSHD- or normal-sized hybrid repeat arrays, respectively [27]. Data from 4 labs on these 'complex' cases shows that they represent between 1% and 5% of cases.

Only 11/19 labs currently use 4qA/B telomeric polymorphism typing, and only in response to a request by the referring clinician for additional testing, and on an individual case basis. The indication is usually in order to check for a proximal (p13E-11 site) deletion in cases believed clinically to have FSHD, or alternatively in prenatal diagnosis where two shortened 4-type fragments are seen in the parents, in order to check if one of these can be discounted from being of relevance if it is of 4qB-type. No lab was yet offering the SSLP 161/163/166 polymorphism, although some may have plans to introduce this on an individual case basis. Similarly, no lab was yet offering any methylation assay to identify cases of FSHD2, as this is not yet felt to be sufficiently discriminatory for service use, although it is hoped that the technical difficulties will be resolved.

The main question of whether to run all available tests (*Eco*RI, *Eco*RI/*Bln*I, 4qA, 4qB, SSLP) on each sample, or whether to use a staged approach, reserving the additional tests for those where they can help answer a particular question, is ongoing. The answer to this is also not one to be set in stone, as it may change with future advances in diagnostic techniques.

10. Consensus genetic testing

At the end of the meeting all participants together discussed the consensus for the molecular diagnosis of FSHD. It was concluded that for know the most suitable method would be the conventional Southern blot based method. For this genomic DNA is digested with *Eco*RI (or *Eco*RI/*Hin*dIII), *Eco*RI/*Bln*I and *Xap*I and the DNA is separated by either LGE or PFGE. After Southern blotting the hybridization is performed with probe p13E-11. Because of the complex diagnostic results that can be encountered, all participants agreed that it would be helpful to know at the time of referral of DNA to the lab, what the clinician's expectation would be for the likelihood of the patient having true FSHD. Based on this short clinical description the geneticist can decide whether to perform additional genetic analysis, or to forward on the sample to a more specialist lab for further DNA tests. In the flowchart presented in Fig. 3, additional analysis are suggested in case the genetic analysis based on the minimum recommended consensus experiments shows an unexpected outcome based on the clinical description.

11. Workshop Participants

Bert Bakker, Leiden, Netherlands
Judit Balog, Leiden, Netherlands
Sat Batish, Worcester, USA
Rafaëlle Bernard, Marseilles, France
Sander Broekman, Leiden, Netherlands
Pili Camaño, San Sebastián, Spain
Daniel Corona, Córdoba, Argentina
Jessica de Greef, Leiden, Netherlands
Giuliana Galluzzi, Rome, Italy
Jürgen Glas, Aachen, Germany
Kaneko Goto, Tokyo, Japan
Francesco Greco, Modena, Italy
Daisy Haggerty, Newcastle, UK
Simone Hasenmüller, Würzburg, Germany
Yukiko Hayashi, Tokyo, Japan
Pascale Hilbert, Gerpinnes, Belgium
Sibylle Jakubiczka, Magdeburg, Germany
Inger Juncker, Aarhus, Denmark
Kiriaki Kekou, Athens, Greece
Rinse Klooster, Leiden, Netherlands
Yvonne Krom, Leiden, Netherlands
Esther Leshinsky, Holon, Israel
Nicolas Levy, Marseilles, France
Elizabeth McCready, Ottawa, Canada
Gerhard Meng, Würzburg, Germany
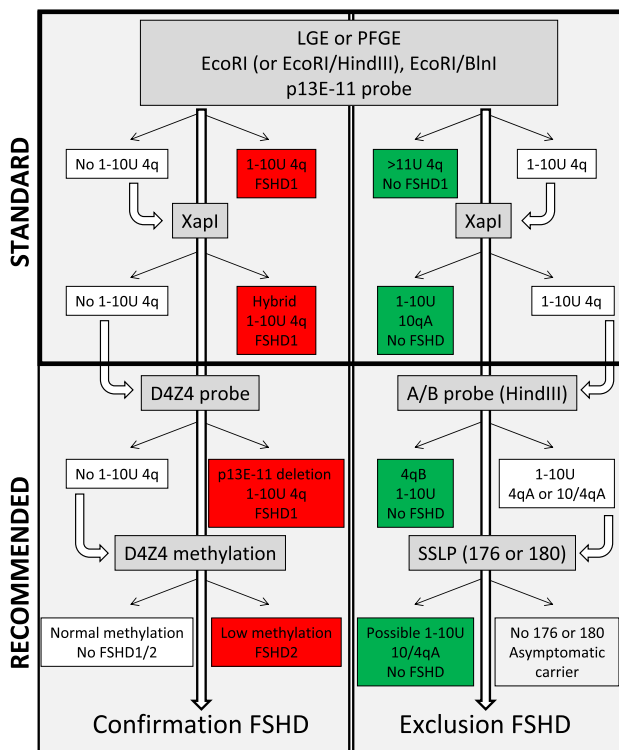
Flowchart molecular diagnosis of FSHD

Fig. 3. Representation of typical Southern blot result of a mosaic FSHD patient (displaying two mosaic alleles, dashed fragments), indicating that the D4Z4 contraction occurred during somatic cell divisions in early embryogenesis.

Henriette Piko, Budapest, Hungary
Sabrina Sacconi, Nice, France
Isabella Scionti, Modena, Italy
Saskia Smith, Leiden, Netherlands
Rossella Tupler, Modena, Italy
Meena Upadhyaya, Cardiff, UK
Michiel van der Wielen, Leiden, Netherlands
Patrick van der Vliet, Leiden, Netherlands
Alan Watts, Sydney, Australia

Acknowledgements

References

[1] Tawil R, van der Maarel S, Padberg GW, van Engelen BG. 171st ENMC international workshop: standards of care and management of facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2010;20:471–5.

[2] Padberg GW. Facioscapulohumeral muscular dystrophy: a clinician's experience. In: Upadhyaya M, Cooper DN, editors. Facioscapulohumeral muscular dystrophy. Clinical medicine and molecular cell biology. Oxon, UK: Garland Science/BIOS Scientific Publishers; 2004. p. 41–54.

[3] van der Maarel SM, Tawil R, Tapscott SJ. Facioscapulohumeral muscular dystrophy and DUX4: breaking the silence. Trends Mol Med 2011;17:252–8.

[4] Lemmers RJ, van der Vliet PJ, van der Gaag KJ, et al. Worldwide population analysis of the 4q and 10q subtelomeres identifies only four discrete interchromosomal sequence transfers in human evolution. Am J Hum Genet 2010;86:364–77.

[5] Bakker E, Wijmenga C, Vossen RH, et al. The FSHD-linked locus D4F104S1 (p13E–11) on 4q35 has a homologue on 10qter. Muscle Nerve 1995;2:39–44.

[6] Lemmers RJ, de Kievit P, Sandkuijl L, et al. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere. Nat Genet 2002;32:235–6.

[7] Lemmers RJ, van der Vliet PJ, Klooster R, et al. A unifying genetic model for facioscapulohumeral muscular dystrophy. Science 2010;329:1650–3.

[8] Lemmers RJ, Wohlgemuth M, Frants RR, Padberg GW, Morava E, van der Maarel SM. Contractions of D4Z4 on 4qB subtelomeres do not cause facioscapulohumeral muscular dystrophy. Am J Hum Genet 2004;75:1124–30.

[9] Lemmers RJLF, van der Wielen MJR, Bakker E, Padberg GW, Frants RR, van der Maarel SM. Somatic mosaicism in FSHD often goes undetected. Ann Neurol 2004;55:845–50.

[10] Lemmers RJ, Osborn M, Haaf T, et al. D4F104S1 deletion in facioscapulohumeral muscular dystrophy: phenotype, size, and detection. Neurology 2003;61:178–83.

[11] van Overveld PG, Lemmers RJ, Sandkuijl LA, et al. Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. Nat Genet 2003;35:315–7.

[12] Goto K, Nishino I, Hayashi YK. Rapid and accurate diagnosis of facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2006;16:256–61.

[13] Nguyen K, Walrafen P, BR, Attarian S, et al. Molecular combing reveals allelic combinations in facioscapulohumeral dystrophy. Ann Neurol, in press. 2011.

[14] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

[15] Lemmers RJLF, van der Wielen MJR, Bakker E, van der Maarel SM. Molecular diagnosis of FSHD. In: Upadhyaya M, Cooper DN, editors. Facioscapulohumeral muscular dystrophy. Clinical medicine and molecular cell biology. Oxon, UK: Garland Science/BIOS Scientific Publishers; 2004. p. 211–34.

[16] Kruchen B, Rueger B. The DIG System–Nonradioactive and Highly Sensitive Detection of Nucleic Acids. BIOCHEMICA 3, 2003.

[17] Deidda G, Cacurri S, Piazzo N, Felicetti L. Direct detection of 4q35 rearrangements implicated in facioscapulohumeral muscular dystrophy (FSHD). J Med Genet 1996;33:361–5.

[18] Lemmers RJL, de Kievit P, van Geel M, et al. Complete allele information in the diagnosis of facioscapulohumeral muscular dystrophy by triple DNA analysis. Ann Neurol 2001;50:816–9.

[19] Matsumura T, Goto K, Yamanaka G, et al. Chromosome 4q;10q translocations; comparison with different ethnic populations and FSHD patients. BMC Neurol 2002;2:7.

[20] van der Maarel SM, Deidda G, Lemmers RJ, et al. De Novo Facioscapulohumeral Muscular Dystrophy: frequent somatic mosaicism, sex-dependent phenotype, and the role of mitotic transchromosomal repeat interaction between Chromosomes 4 and 10. Am J Hum Genet 2000;66:26–35.

[21] Tonini MM, Lemmers RJ, Pavanello RC, et al. Equal proportions of affected cells in muscle and blood of a mosaic carrier of facioscapulohumeral muscular dystrophy. Hum Genet 2006;119:23–8.

[22] Ehrlich M, Jackson K, Tsumagari K, Camano P, Lemmers RJ. Hybridization analysis of D4Z4 repeat arrays linked to FSHD. Chromosoma 2007;116:107–16.

[23] de Greef JC, Lemmers RJ, Camano P, et al. Clinical features of facioscapulohumeral muscular dystrophy 2. Neurology 2010;75:1548–54.

[24] Barat-Houari M, Nguyen K, Bernard R, et al. New multiplex PCR-based protocol allowing indirect diagnosis of FSHD on single cells: can PGD be offered despite high risk of recombination? Eur J Hum Genet 2010;18:533–8.

[25] Lemmers RJ, Wohlgemuth M, van der Gaag KJ, et al. Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy. Am J Hum Genet 2007;81:884–94.

[26] Tsumagari K, Chen D, Hackman JR, Bossler AD, Ehrlich M. FSH dystrophy and a subtelomeric 4q haplotype: a new assay and associations with disease. J Med Genet 2010;47:745–51.

[27] Buzhov BT, Lemmers RJ, Tournev I, et al. Genetic confirmation of facioscapulohumeral muscular dystrophy in a case with complex D4Z4 rearrangments. Hum Genet 2005;116:262–6.