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Molecular combing compared to Southern blot for measuring D4Z4 contractions in FSHD

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Abstract

We compare molecular combing to Southern blot in the analysis of the facioscapulohumeral muscular dystrophy type 1 locus (FSHD1) on chromosome 4q35-qter (chr 4q) in genomic DNA specimens sent to a clinical laboratory for FSHD testing. A de-identified set of 87 genomic DNA specimens determined by Southern blot as normal (n = 71), abnormal with D4Z4 macrosatellite repeat array contractions (n = 7), indeterminate (n = 6), borderline (n = 2), or mosaic (n = 1) was independently re-analyzed by molecular combing in a blinded fashion. The molecular combing results were identical to the Southern blot results in 75 (86%) of cases. All contractions (n = 7) and mosaics (n = 1) detected by Southern blot were confirmed by molecular combing. Of the 71 samples with normal Southern blot results, 67 (94%) had concordant molecular combing results. The four discrepancies were either mosaic (n = 2), rearranged (n = 1), or borderline by molecular combing (n = 1). All indeterminate Southern blot results (n = 6) were resolved by molecular combing as either normal (n = 4), borderline (n = 1), or rearranged (n = 1). The two borderline Southern blot results showed a D4Z4 contraction on the chr 4qA allele and a normal result by molecular combing. Molecular combing overcomes a number of technical limitations of Southern blot by providing direct visualization of D4Z4 macrosatellite repeat arrays on specific chr 4q and chr 10q alleles and more precise D4Z4 repeat sizing. This study suggests that molecular combing has superior analytical validity compared to Southern blot for determining D4Z4 contraction size, detecting mosaicism, and resolving borderline and indeterminate Southern blot results. Further studies are needed to establish the clinical validity and diagnostic accuracy of these findings in FSHD.

Keywords: Muscular dystrophy; Facioscapulohumeral; Blotting; Southern; Electrophoresis; Gel; Pulsed-field; Gene mapping; Chromosome mapping; In situ hybridization; Fluorescence

1. Introduction

Facioscapulohumeral dystrophy (FSHD) is a progressive myopathy [1] that exhibits autosomal dominant inheritance in affected families. Roughly one-fourth of cases are sporadic or mosaic [2,3]. Weakness is often asymmetric, with initial involvement of the facial muscles followed by progressive dystrophy of the scapular, humeral, truncal, and lower extremity muscles. The sparing of the extraocular and bulbar muscles and the distinct topography of the myopathy result in a characteristic facies and body habitus characterized by normal eye movements with difficulty in closing the eyelids, a transverse smile, pectoralis major atrophy, scapular winging, pronounced lumbar lordosis with a protuberant abdomen, and a steppage gait. The disease onset is usually before the second decade of life with variable severity, progression, and extramuscular manifestations [4]. Molecular testing identifies at least two forms, FSHD1 (OMIM 158900) and FSHD2 (OMIM 158901), which are clinically indistinguishable from one another [5]. FSHD1 is the most prevalent form and occurs in approximately 95% of FSHD cases, whereas FSHD2 occurs in less than 5%

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of affected individuals [6]. The FSHD1 locus on chromosome 4q35-qter (chr 4q) [7–9] contains a highly polymorphic 3.3 kilobase (kb) tandem macrosatellite repeat array called D4Z4. Normal individuals have 11 to over 100 copies of these D4Z4 arrays. In contrast, individuals with FSHD1 have D4Z4 copy numbers within the pathogenic range with as few as one to 10 repeats [10].

Molecular analysis of the FSHD1 locus is complicated by the presence of a highly homologous region on the subtelomere of chr 10q26 [11–14]. Further complexity arises due to the existence of two alternative chr 4q alleles designated as chr 4qA and 4qB. The chr 4qA allele contains a polyadenylation site for the transcription factor double homeobox protein 4 (DUX4) and is referred to as the permissive locus. A decrease in copy number (i.e. contraction) of the D4Z4 repeats to within the pathogenic range on 4qA subtelomeres permits the expression of functional DUX4, which in turns triggers the progressive muscle degeneration in FSHD1 [15–17]. In contrast, D4Z4 contractions on 4qB subtelomeres do not cause FSHD1.

The mechanism of disease in FSHD2 does not involve D4Z4 repeat contraction. However, both FSHD1 and FSHD2 share a common molecular pathogenesis that involves insufficient silencing of the transcription factor *DUX4* within the D4Z4 macrosatellite repeat [18–20]. This occurs either by D4Z4 contractions in FSHD1 [21–26] or by heterozygous mutations in the structural maintenance of chromosomes hinge domain-containing protein 1 gene, *SMCHD1*, on chr 18 in FSHD2 [19,27,28].

Southern blot analysis (SB) has been the standard laboratory diagnostic method used to detect FSHD1-related contractions of D4Z4 on chr 4. *Eco*RI restriction enzyme digestion of genomic DNA, followed by SB analysis using a specific probe (p13E-11) proximal to the D4Z4 array, detects repeats on both chr 4 and chr 10, as well as a homologous 9.5 kb region on chr Y [10]. Despite the high sequence homology between the D4Z4 repeats on chr 4 and chr 10, there are distinct polymorphic differences. Each of the chr 10 specific D4Z4 repeats contains a *Bln*I restriction enzyme site that is absent on repeats derived from chr 4. Thus, chr 4 and chr 10 alleles are distinguished by SB using a double restriction enzyme digestion with *Eco*RI and *Bln*I [29]. *Bln*I digestion leaves the chr 4q D4Z4 repeat units intact while fragmenting the chr 10q sites. *Bln*I digestion also shifts the chr 4q *Eco*RI fragment by approximately 3 kb–5 kb (Fig. 1A) [3,10,29].

Current diagnostic testing for FSHD1 by SB may lead to indeterminate results in up to 23% of cases [30]. Technological limitations are evident when a deletion of the genomic sequence upstream of the chr 4 D4Z4 repeat units includes the p13E-11 probe region [31,32] and in cases with somatic mosaicism (i.e. cells with two different genotypes) or rearrangements [33]. DNA molecular combing (MC) hybridizes multi-color DNA probes onto uniformly stretched DNA fiber and accurately identifies the 4qA and other alleles [34]. The precise measurement of the D4Z4 repeat motif by DNA combing may help correlate the size of the contracted 4qA allele with clinical features. In this study, we compare SB with molecular combing (MC), and show that MC represents an improvement over SB by its analytical ability to directly visualize D4Z4 macrosatellite repeat arrays on specific chr 4q and chr 10q alleles, detect mosaicism and rearrangements, and provide more precise D4Z4 repeat sizing.

2. Materials and methods

2.1. Patient specimens

Eighty-seven anonymous, de-identified genomic DNA specimens referred previously for SB testing for FSHD1 at the Athena Diagnostics laboratory were analyzed in a blinded fashion by MC at the cytogenetics laboratory at Quest Diagnostics Nichols Institute. The SB patient specimen results consisted of normal (n = 71), D4Z4 contractions (n = 7), borderline (n = 2), indeterminate (n = 6), and mosaic (n = 1). Affected FSHD1 cell lines (Coriell Institute, Camden, New Jersey) were used as positive controls. All of the patient specimens were coded and the SB results blinded prior to MC analysis.

2.2. Pulsed field gel electrophoresis of the 4q35 and 10q26 alleles and Southern blotting

SB was performed as described previously [35] with the following modifications. Intact white blood cells were separated from anti-coagulated whole blood by a Ficoll gradient. Intact cells were mixed at a predetermined concentration with low melt agarose and specimens were placed into the chambers of a mold and cooled at room temperature. The agarose plugs were removed from the mold, incubated with detergents and enzymes, and washed to remove all of the cellular components from the DNA. The agarose plugs were incubated with the restriction enzyme, *Eco*RI alone, and with *Eco*RI and *Bln*I for a double restriction enzyme digestion. The DNA from the *Eco*RI digest and the double digest with *Eco*RI and *Bln*I was resolved in side-by-side lanes by pulsed field electrophoresis (PFGE), denatured and transferred to a nylon membrane. A p13E-11 labeled probe [10] was cloned independently and labeled by chemiluminescence. Specimens were compared to molecular weight markers (BioRad, Hercules CA) and FSHD1 positive control cell lines. Patient results were interpreted based on the migration of allele pairs as follows: normal: ≥42 kb, borderline: 38–41 kb, FSHD1 contraction: 12–37 kb, and indeterminate: atypical migration pattern or not interpretable (Fig. 1).

2.3. DNA molecular combing of the 4q35 and 10q26 alleles

The FSHDCombing Test™ was performed as described previously [34] with the following modifications. Agarose plugs were melted and digested with β-agarase overnight. High molecular weight (>500 kb) DNA was isolated by overnight treatment with proteinase K and sarkosyl followed by extensive washing with buffer. Silane-coated glass coverslips were inserted into a purified DNA solution in a 2-(N-morpholino) ethanesulfonic acid sodium buffer using a motorized platform for DNA combing (Genomic Vision, France). The coverslip was pulled out of the solution at 300 μm/s. The DNA was stretched uniformly to a length of 1 μm per 2 kb by the action of the receding meniscus. After combing, the DNA was bound to the

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