Strategy for mutation analysis in the autosomal recessive limb-girdle muscular dystrophies

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Abstract

We describe a strategy for molecular diagnosis in the autosomal recessive limb-girdle muscular dystrophies, a highly heterogeneous group of inherited muscle-wasting diseases. Genetic mutation analysis is directed by immunoanalysis of muscle biopsies using antibodies against a panel of muscular dystrophy-associated proteins. Performing the molecular analysis in this way greatly increases the chance that mutations will be found in the first gene examined. The use of this strategy can significantly decrease the time involved in determining the genetic fault in a patient with a clinical diagnosis of recessive limb-girdle muscular dystrophy, as well as having a feedback effect, which is useful in helping clinicians to identify subtle clinical differences between the subtypes of the disease. The use of this approach has so far helped us to identify mutations in ten sarcoglycanopathy (limb-girdle muscular dystrophy 2C–2F) patients, and seven calpainopathy (limb-girdle muscular dystrophy 2A) patients. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The limb-girdle muscular dystrophies (LGMD) are a highly heterogeneous group of progressive muscle wasting diseases, which may be inherited in either an autosomal dominant (LGMD type 1) or autosomal recessive (LGMD type 2) fashion [1]. Of the three dominant forms so far identified [2–4], two of the genes have been identified; the lamin A/C gene responsible for LGMD 1B (also responsible for autosomal dominant Emery–Dreifuss muscular dystrophy) [5,6], and the caveolin-3 gene responsible for LGMD 1C [7].

The recessive forms are even more heterogeneous, with nine genetically distinct forms identified (LGMD 2A–2I) [8–17]. At the molecular level, they can be divided into the sarcoglycanopathies [11,18–20] and the non-sarcoglycanopathies [21–23], based on whether they are caused by mutations in a gene encoding a member of the sarcoglycan (SG) component of the dystrophin-associated protein complex or not. This complex is located at the muscle cell membrane, and is thought to provide a mechanical link between the cell cytoskeleton and the extracellular matrix [24]. The sarcoglycans (α-, β-, γ- and δ-SG) form a subcomplex whose components are interdependent to varying degrees. Evidence suggests that β- and δ-SG are most tightly associated together, with γ-SG less tightly bound, and α-SG is the most loosely associated member of the complex [25]. In most cases, mutations in one of the sarcoglycan genes causes a secondary reduction of the other three proteins that can vary from partial deficiency to total absence [26].

The non-sarcoglycanopathies, as their name implies involve proteins which are not members of the sarcoglycan complex. Of the five loci currently known (LGMD 2A, 2B, 2G–2I), two genes have so far been isolated, those of the muscle-specific calpain (CAPN3) [21] responsible for LGMD 2A, and the dysferlin gene [22,23] responsible for LGMD 2B and its allelic variant Miyoshi myopathy. The
functions of both these genes and their proteins are currently being investigated. In general, the non-sarcoglycanopathies show a later onset and milder disease progression than the sarcoglycanopathies, though many exceptions have been reported [27].

The level of genetic heterogeneity seen in this group of diseases, together with the possibility of clinical overlap with other neuromuscular disorders demands that the approach to mutation analysis be as efficient as possible in terms of both time and money, if LGMD analysis is to be introduced into the diagnostic setting. Detection of the mutation, and thereby confirmation of the primary molecular pathological event in any particular patient is necessary for absolute diagnosis, but specifically for genetic counseling or prenatal diagnosis. Similarly, any gene-based therapy will depend upon knowledge of the mutation. All of the recessive LGMD genes cloned so far are multi-exonic, with few, if any recurrent mutations. Here we present a strategy for immunologically guided mutation analysis that greatly increases the chance that mutations will be found in the first gene examined in any particular case. We illustrate our approach by describing some results of our own analyses in the sarcoglycans and CAPN3.

2. Materials and methods

2.1. Patients

The clinical diagnostic criteria for the recessive limb-girdle muscular dystrophies have been comprehensively reviewed by [27].

Thirteen patients were selected for mutation analysis in the CAPN3 gene, based on the criteria that they had abnormal labelling of the calpain 3 protein on western blots, and normal dysferlin labelling. Fourteen patients had abnormal labelling for the sarcoglycans and were selected for mutation analysis in the sarcoglycan genes.

Patients with abnormal dysferlin labelling are the subject of a further paper (Harrison et al., in preparation).

2.2. Immunolabelling

Immunolabelling of tissue sections and blots was performed as described previously [28,29]. Muscle biopsy samples are examined using the antibodies listed in Table 1. This paper is specifically concerned with antibodies relating to diagnosis of AR LGMD. However, a much wider range of antibodies is routinely used in examination of biopsies from patients with unknown muscle problems (Table 1).

2.3. Genetic mutation analysis

A combination of single strand conformational polymorphism (SSCP) analysis and heteroduplex analysis (HA) was used as described previously [30]. The four sarcoglycan genes between them are made up of 31 exons, with CAPN3 and the dysferlin gene adding a further 79.

3. Results

3.1. The sarcoglycanopathies

Fourteen patients presented with a protein profile indicative of primary mutations in one of the sarcoglycan genes (Table 2). Of these, five individuals (γ1–γ5) appeared to have γ-sarcoglycan more severely reduced than the other three proteins (Fig. 2B,D). These five patients were there-

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Antibodies routinely used in analysis of muscle biopsies*</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Dystrophin (N-term)</td>
</tr>
<tr>
<td>Dystrophin (rod)</td>
</tr>
<tr>
<td>Dystrophin (C-term)</td>
</tr>
<tr>
<td>α-Sarcoglycan</td>
</tr>
<tr>
<td>β-Sarcoglycan</td>
</tr>
<tr>
<td>γ-Sarcoglycan</td>
</tr>
<tr>
<td>δ-Sarcoglycan</td>
</tr>
<tr>
<td>Calpain 3 (exon 1)</td>
</tr>
<tr>
<td>Calpain 3 (exon 8)</td>
</tr>
<tr>
<td>Dysferlin</td>
</tr>
<tr>
<td>Laminin 2 chain (80 kDa)</td>
</tr>
<tr>
<td>Laminin α-2 chain (300 kDa)</td>
</tr>
<tr>
<td>Caveolin 3</td>
</tr>
<tr>
<td>Emerin</td>
</tr>
<tr>
<td>β-Spectrin</td>
</tr>
<tr>
<td>β-Dystroglycan</td>
</tr>
<tr>
<td>Laminin α-5 chain</td>
</tr>
<tr>
<td>Laminin β-1 chain</td>
</tr>
<tr>
<td>Laminin γ-1 chain</td>
</tr>
</tbody>
</table>

* All antibodies from Novocastra except * Chemicon and ** Transduction laboratories. M, monoclonal.
fore examined for mutations in the γ-sarcoglycan gene. At least one pathogenic sequence alteration was found in each of the five patients (Table 2). One of the patients (γ-4, Fig. 2D) showed labelling for sarcoglycan, which was indistinguishable from normal. This strongly indicates that the use of the α-sarcoglycan antibody alone is insufficient for distinguishing between sarcoglycanopathies and non-sarcoglycanopathies (see Table 2 and Fig. 2).

It has been reported that individuals with mutations in the β- and δ-sarcoglycan genes show severe reduction of the entire complex [19,26]. Four patients with such a profile were first examined for mutations in the β-sarcoglycan gene. Mutations were found in two of these individuals, including one patient with cardiomyopathy, discussed in [31], but the others showed no mutations in either β- or δ-sarcoglycan.

Three individuals with variable levels of reduction in the components of the complex showed mutations in the α-sarcoglycan gene. One of the mutations seen was the highly recurrent Arg77Cys, seen in at least 32% of sarcoglycanopathy chromosomes [32,33]. The α-sarcoglycan protein levels in these patients varied from moderately reduced to completely absent (Table 2).

In total, four patients with sarcoglycanopathy protein profiles showed no mutation in any of the sarcoglycan genes examined.

### 3.3. LGMD 2A

Thirteen patients showed normal dysferlin but reduced or absent labelling for the calpain 3 protein on Western blots (Fig. 3). This indicated that the primary genetic defect in these individuals might be in the CAPN3 gene. Sarcoglycan labelling was normal for all of these patients.

Of these patients, eight have been genetically confirmed as calpainopathy cases. No mutations were found in five cases after all the exons and the promoter had been examined. The second mutation was not found in four cases (see Table 3).

Dysferlin mutation analysis is discussed in (Harrison et al., in preparation).

### 3.4. Development of the integrated approach to diagnosis of LGMD

Fig. 1 shows a flow diagram representing the development of our approach to finding mutations in patients with a possible diagnosis of recessive LGMD. The first distinction that is made is whether or not dystrophin is present at normal abundance. Biopsies with reduced abundance of full size dystrophin which do not show small deletions in the dystrophin gene are examined with the sarcoglycan antibodies to look for sarcoglycan profiles before attempting to search for point mutations in the dystrophin gene. In BMD muscle, dystrophin is more severely reduced than the sarcoglycans, which are seen at variable levels. Also, in BMD, dystrophin reduction is seen with antibodies to both the N- and C-terminals, whereas if dystrophin is reduced in sarcoglycanopathy (Anderson, personal communication), it is often seen to be reduced with the C-terminal antibody only. Assessment of clinical information should also be taken into account [27].

Having eliminated the samples with primary dystrophinopathy, attention is focused on the six known recessive LGMD proteins.

On the sarcoglycanopathy side, the most distinctive profile in our patient population is associated with

### Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Protein profile</th>
<th>Mutation</th>
<th>Protein change</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-1</td>
<td>α-, β+, γ-, δ+, dystrophin reduced</td>
<td>229C &gt; T</td>
<td>R77C</td>
</tr>
<tr>
<td>α-2</td>
<td>α-, β-, γ-, δ+</td>
<td>266/267TC &gt; CT, 850C &gt; T</td>
<td>L89P, R284C</td>
</tr>
<tr>
<td>α-3</td>
<td>α-, β+, γ-, δ+</td>
<td>409G &gt; A (homozygous)</td>
<td>Q137K</td>
</tr>
<tr>
<td>β-1</td>
<td>α-, β-, γ+, δ+</td>
<td>82-86 del GAGAG (homozygous)</td>
<td>Stop 32</td>
</tr>
<tr>
<td>β-2</td>
<td>α-, β-, γ+, δ+</td>
<td>341C &gt; T, 499G &gt; A</td>
<td>S114F, G167S</td>
</tr>
<tr>
<td>γ-1</td>
<td>α++, β++, γ-, δ+</td>
<td>521delT</td>
<td>Stop 193</td>
</tr>
<tr>
<td>γ-2</td>
<td>α++, β++, γ-, δ+</td>
<td>IVS3 + 6 A &gt; T, 581T &gt; C</td>
<td>? Splicing, L194S</td>
</tr>
<tr>
<td>γ-3</td>
<td>γ-sarcoglycan absent, others severely reduced</td>
<td>206G &gt; C</td>
<td>G69R</td>
</tr>
<tr>
<td>γ-4</td>
<td>α++, β+, γ-, δ+</td>
<td>582InsA (homozygous)</td>
<td>Stop 219</td>
</tr>
<tr>
<td>γ-5</td>
<td>α++, β++, γ-, δ+</td>
<td>IVS2 + 2 del 4bp</td>
<td>? Splicing</td>
</tr>
</tbody>
</table>

* Patients α-1–α-3, LGMD 2D; patients β-1 and β-2, LGMD 2E; patients γ-1–γ-5, LGMD 2C. ++, Normal; +, slightly reduced; +/−, reduced; −, absent. Biopsy section analysis of patients α-1, γ-1 and γ-4 are seen in Fig. 2C,B and D, respectively.
LGMD 2C, where total loss of γ-sarcoglycan is seen in most cases, with the others present at variable levels. Patients showing variable labelling for the complex, or severely reduced labelling for α-SG with variable levels of the others, are referred for α-sarcoglycan gene analysis in the first instance, starting with the common C229T mutation which can be detected by restriction analysis of the PCR product. Those showing severe reduction of the entire complex are most likely to have β- or δ-sarcoglycan mutations. The latter are referred for β-sarcoglycan analysis in the first instance since mutations in the δ-sarcoglycan gene are rare.

Antibodies are available to the proteins involved in LGMD 2A (calpain 3) and LGMD 2B (dysferlin) [34,35]. Two antibodies against epitopes in the muscle-specific calpain protein are used on western blots to determine relative abundance of this protein in the patient’s muscle. Abnormal labelling is an indication of primary calpainopathy (LGMD 2A). However, dysferlin immunoanalysis should be performed in parallel, since it has recently been shown that a proportion of LGMD 2B patients can show a secondary reduction in labelling for the muscle specific calpain protein (Anderson et al., in press). This secondary reduction, however, is not as severe as the dysferlin reduction seen. Dysferlin reduction has not been seen in calpainopathy patients.

Those patients showing reduced levels of the dysferlin protein on blots (including any who also have calpain 3 reduction) are referred for mutation analysis in the DYSF gene.

In all cases, where mutations are not seen in the first gene examined, one should consider examining the remaining sarcoglycan genes (or non-SG genes in the case of non-sarcoglycanopathy).

### 4. Discussion

The highly heterogeneous nature of the autosomal recessive limb-girdle muscular dystrophies demands that an efficient strategy be developed to direct the process of searching for genetic mutations within the six known genes. We suggest that complete examination of the protein profile (in combination with a comprehensive clinical examination) is an excellent starting point for the determination of genetic diagnosis in LGMD.

The strategy presented here is intended to provide the best advice on where one should begin the process of mutation analysis in LGMD, and is designed to increase the chance that mutations will be found in the first gene analyzed. As with many aspects of neuromuscular disease, it is likely that exceptions will exist. If the mutations are not seen in the first
gene, it will be necessary to examine the other possible candidates.

For routine biopsy examinations, we use a wide range of antibodies, as listed in Table 1 [36]. In particular, labelling with antibodies to the laminin chains \(\alpha-2\) (merosin), \(\alpha-5\), \(\beta-1\) and \(\gamma-1\) is undertaken. Patients with clinical diagnosis of LGMD have been reported with mutations in the LAMA2 gene [37]. In addition, a secondary reduction in laminin \(\beta-1\) labelling may occur in some dominant LGMD patients with primary mutations in the lamin A/C and collagen VI genes [38,39]. A group of adult LGMD patients has been described who have abnormal labelling for laminin \(\alpha-2\) on blots, but the primary gene defect in these patients is unknown [40]. Immunohistochemical patterns in the sarcoglycanopathies are becoming ever better characterized. Originally, it was believed that mutations in one of the sarcoglycan genes led to non-specific reduction of the entire complex, and so only the \(\alpha\)-sarcoglycan antibody was used to distinguish sarcoglycanopathies from other forms of LGMD. However, it is now clear that the different sarcoglycanopathies can have distinct protein profiles when examined with all four antibodies. The importance of using antibodies to all of the proteins is emphasized by the case of patient \(\gamma-4\), who had a homozygous truncation mutation in the \(\gamma\)-sarcoglycan gene, and showed complete absence of the protein, but whose labelling for \(\alpha\)-sarcoglycan appeared normal. This patient may not have been referred for sarcoglycan gene analysis if the \(\alpha\)-sarcoglycan antibody alone had been used for immunohistochemistry.

That \(\alpha\)- and \(\gamma\)-SG do not have so great an effect on the other members of the complex may be explicable due the fact that they are less closely associated than are \(\beta\)- and \(\delta\)-SG [25].

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**Fig. 2.** Immunological analysis of muscle biopsy sections. (A): LGMD 2A, (B): LGMD 2C, patient \(\gamma\)-1, (C): LGMD 2D, patient \(\alpha\)-1, (D): LGMD 2C, patient \(\gamma\)-4 and dystrophin-C: antibody to dystrophin C-terminus.
The components of the dystrophin-associated protein complex appear to be more dependent on dystrophin than dystrophin is on the complex, and therefore abnormal dystrophin labelling is most often indicative of either Duchenne or Becker muscular dystrophy, and samples are referred for DNA analysis accordingly. As is often the case in neuromuscular disease, this is the general rule, however exceptions do exist. We and other groups [41] have found patients whose protein profile indicates X-linked muscular dystrophy due to reduced dystrophin labelling, but who have later been shown to have disease-causing mutations in one of the sarcoglycan genes. These results, may suggest that the relationship between dystrophin and the sarcoglycans is more complex than previously thought.

Knowledge of calpain 3 is still very incomplete. Little is known about its functions and interactions with other proteins [42,43]. The matter is further complicated by the fact that calpain 3 is a proteolytic enzyme, and is known to be very prone to undergo autolysis, something which causes a risk of false-positive results. Also, recent evidence indicates that some patients with primary dysferlinopathy (LGMD 2B) can exhibit reduction of calpain 3 as a secondary effect (Anderson et al., in press). It is therefore imperative that a patient showing reduced levels of calpain also be examined with the dysferlin antibody before being referred for mutation analysis. Also, clinical information is extremely important in distinguishing LGMD 2A from LGMD 2B/MM (Pollitt et al., in press).

Though the correlation is not yet 100% effective, the results presented here validate the use of antibodies against all of the known LGMD proteins for directing mutation analysis.

This process can, where possible be complemented by microsatellite analysis, as shown (Table 3). Where families are not large enough for linkage, haplotypes may still be useful in excluding candidate loci.

It is also worth while taking into account such factors as common mutations. The C229T mutation in α-sarcoglycan which accounts for at least 32% of mutated α-SG chromosomes can be rapidly detected using an NlaIV digestion [32,33], and is therefore a test worth performing first in samples thought to have α-sarcoglycanopathy. If a sample comes from North Africa, and is thought to be a γ-sarcoglycanopathy sample, one should examine the microsatellite D13S232 for the 122 base-pair disequilibrium allele, which is associated with the delT521 ‘Tunisian' mutation [44].

In a number of cases, particularly those with calpain 3 reduction, no mutations were found. There are a number of

![Fig. 3. Western blot analysis showing reduced labelling for the calpain 3 protein. Lane 1: calpain 3 reduction, mutations unknown, lane 2: patient C-2, lane 3: patient C-4, lane 4: patient C-3. C: control, Dys, dystrophin; Dysf, dysferlin; α-SG, α-sarcoglycan; β-DG, β-dystroglycan and MHC; myosin heavy chain (loading control).](image-url)

**Table 3**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Calpain profile</th>
<th>Mutations</th>
<th>Protein change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>94 +, 30 −, 60 −</td>
<td>1435A &gt; G</td>
<td>S479G</td>
</tr>
<tr>
<td>C-2</td>
<td>94 +, 30 −, 60 −</td>
<td>550delA, 2114A &gt; G</td>
<td>Stop 189, D705G</td>
</tr>
<tr>
<td>C-3</td>
<td>94 −, 30 −, 60 −</td>
<td>1373delC, 1983delA</td>
<td>Stop 462, stop 667</td>
</tr>
<tr>
<td>C-4</td>
<td>94 + +, 30 +, 60 +</td>
<td>649G &gt; A, 2093G &gt; C</td>
<td>E217K, R698P</td>
</tr>
<tr>
<td>C-5</td>
<td>94 −, 30 −, 60 −</td>
<td>550delA</td>
<td>Stop 189</td>
</tr>
<tr>
<td>C-6</td>
<td>94 +, 30 −, 60 −</td>
<td>566T &gt; C, IVS18-1 G &gt; T</td>
<td>L189P, splicing</td>
</tr>
<tr>
<td>C-7</td>
<td>94 −, 30 −, 60 +</td>
<td>328C &gt; G</td>
<td>R110X</td>
</tr>
<tr>
<td>C-8</td>
<td>Ascertained by linkage</td>
<td>2362AG &gt; TCATCT</td>
<td>Stop 788</td>
</tr>
</tbody>
</table>

a 94, Full length calpain 3 protein; 30, 30 kDa fragment; 60, 60 kDa fragment. + + +, Normal; + +, slightly reduced; +, very reduced; +/−, severely reduced; −, absent.

b This patient’s protein profile can be seen in Fig. 2, lane 2.

c This patient’s protein profile can be seen in Fig. 2, lane 4.

d This patient’s protein profile can be seen in Fig. 2, lane 3.
possible reasons for this. First, there may be mutations present, which would not be detected by the techniques used. These include large-scale deletions, mutations deep into the introns, or in unexamined areas of the promoter or enhancer regions [45]. Second, mutations in as yet unidentified genes may cause secondary reduction of the sarcoglycan complex, or calpain 3 [46,47]. Third, in the case of calpain reduction, it is possible that handling of biopsies, something which is often out of our control, might subject the tissue to conditions which promote autolysis.

While it is possible that false positives for calpainopathy may be seen, (i.e. calpain reduction despite no mutations in the gene), no evidence has yet indicated that false negatives may exist, i.e. all individuals known to have mutations in the CAPN3 gene also show calpain 3 reduction on blots (even if it is only the lower 30 and 60 kDa bands).

The two individuals who had severe reduction of the entire sarcoglycan complex but did not have show mutations in either the β- or δ-sarcoglycan genes may yet turn out to have mutations in either α- or γ-sarcoglycan. This is under investigation.

This approach to diagnosis of LGMD is constantly being developed. As more samples are characterized at the genetic level, one can then look back at the immunohistochemical profiles and further refine the distinguishing features of the different subtypes of the disease, with a view toward drawing correlations between protein profiles and the types of mutation. This is also true of the clinical evaluation of patients, whereby clinicians may pick out subtle features, which distinguish one form of LGMD from another. This multidisciplinary feedback is critical to the development of diagnostics as our understanding of this group of neuromuscular disorders develops.

Acknowledgements

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References


