Original Article

Diagnosis of limb-girdle muscular dystrophy 2A by immunohistochemical techniques

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The Western blot technique is currently the standard detection method for suspected limb girdle muscular dystrophy (LGMD) 2A (calpainopathy). This is the first report in the English literature of the successful application of immunohistochemical techniques to support a diagnosis of LGMD 2A. This approach is straightforward and appears to be reasonably specific. We propose that immunohistochemical methods should be re-evaluated for the screening of undiagnosed patients with suspected LGMD 2A.

Key words: calpain-3, immunohistochemistry, limb girdle muscular dystrophy 2A, myopathy, Western blot.

INTRODUCTION

Limb girdle muscular dystrophy (LGMD) 2A is caused by mutations in the calpain-3 (CAPN3) gene (also called p94 gene), which encodes CAPN3, the large skeletal-muscle-specific member of the calpain family.1 It has been reported to be the most frequent autosomal recessive form of muscular dystrophy in many populations.2–6

Most limb girdle muscular dystrophies are screened using immunohistochemical techniques, including the sarcoglycanopathies and merosin-deficient muscular dystrophies. However, the Western blot technique is currently the accepted detection method for suspected LGMD 2A (calpainopathy). This, at least in part, derives from the perception that calpain-3 antibodies do not work on immunochemistry.7,8

To the best of our knowledge, this manuscript is the first description in the English literature demonstrating that LGMD 2A can be effectively screened using immunohistochemical staining with commercially available calpain antibodies. There is one previous case report written in Japanese with successful application of immunostaining with a different monoclonal antibody against human calpain-3.9

METHODS

We systematically identified a cohort of 21 patients with proximal limb-girdle weakness and dystrophic changes on muscle biopsy. Appropriate ethical approval and informed consent were obtained, in accordance with the Declaration of Helsinki.

Total protein was extracted from available muscle samples. Cell lysis was performed at boiling temperature using 20 mM Tris-HCl, pH 7.4, containing 2% SDS, 5 mM ethyleneglycoltetraacetic acid (EGTA), 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg leupeptin/mL and 10 μg aprotinin/mL. Measured aliquots of the extracted proteins were subjected to SDS-PAGE (7.5% acrylamide). Western-blot was performed using calpain-3 monoclonal antibodies calp3d/12A2 and calp3d/2C4 (Novocasta Laboratories (NCL), Newcastle-upon-Tyne, UK), and the reaction was detected by using horseradish peroxidase-conjugated anti-IgG (1 U/mL; Boehringer) and staining with diaminobenzidine. The dilutions for NCL-CALP-2C4 were 1:25–1:50 and for NCL-CALP-12A2, 1:100.

The muscle cryosections of all limb-girdle patients were then subjected to immunohistochemical staining as follows: 7-μm frozen tissue sections were mounted on positively charged microscope slides, air dried, and fixed in acetone for 10 min at room temperature. The sections were
then incubated at room temperature with a 1:50 dilution of mouse antihuman calpain-3, clone 12A2 (Novocastra) for 60 min. Subsequent immunodetection was done using the Vector Elite ABC detection system (Vector Laboratories, Burlingame, CA, USA) according to manufacturer’s instructions. Sections were counterstained with hematoxylin for nuclear detail. Appropriate positive controls (normal muscle) and negative controls were also performed in parallel.

Mutation screening of the CAPN3 gene was performed using the single strand conformation polymorphism (SSCP) technique for confirmation in suspected cases. DNA samples were amplified by PCR. Upon screening of the calpain-3 gene through SSCP, samples with abnormal migration pattern on SSCP gel were sequenced in an automatic sequencer machine (ABI Model 377 Version 3.0).

RESULTS

We identified three patients with progressive predominantly proximal limb-girdle weakness and dystrophic muscle biopsies. In these cases, Western blots demonstrated an absence/marked paucity of antibody staining with the calpain 2C4 (exon 1) and 12A2 (exon 8) antibodies (Fig. 1A,B, respectively). This involved the 94 kDa band, as well as the 60 and 30 kDa bands. All patients had clinical findings consistent with LGMD 2A as previously described.1,10,11

There was concordant absence of immunohistochemical 12A2 calpain antibody staining in all three of these muscle biopsy specimens (Fig. 2). Of note, calpain-3 was immunopositive in control muscles, as well as in other types of limb-girdle muscular dystrophies.

In one patient, SSCP and screening of all coding regions of the calpain-3 gene revealed a heterozygous 550delA mutation (exon 4) but the second mutation was not identified, even after direct gene sequencing. In a second patient, DNA samples could not be obtained, while in the third one the DNA quality did not allow its amplification.

The patient who had concordant laboratory evidence of calpainopathy presented with acquired progressive ambulatory difficulties at 6 years of age. This included frequent falls and problems with climbing stairs. At age 12, he was ambulant but relied on using the rails with stairs. On examination, he demonstrated scapular winging, decreased bulk in the upper arms, calf hypertrophy, and mild asymmetrical heel cord contractures. Gower’s sign was absent. However, by MRC (Medical Research Council) scale measurements, he had hip- more than shoulder-girdle weakness (4/5), disproportionately weak hip extensors (2/5), and mild neck flexor weakness. Deep tendon reflexes were absent in upper extremities, but normal in lower extremities. Creatine phosphokinase ranged from 7744 to 10 770 U/L. There were mild dystrophic changes in the right triceps muscle biopsy. By age 17 years he had progressed, with a full Gower’s sign. Quadriceps were 2/5 and 2+/5 (right and left, respectively). Hip extensors were 2/5 and hamstrings 3/5. Pulmonary function tests were normal. Cardiac evaluation was normal including echocardiogram. His Western blot demonstrated absent staining of the
94 kDa band with the calpain 2C4 antibodies and faint staining with the 12A2 antibodies (Fig. 1A,B – lane 10). Immunohistochemical staining with the 12A2 antibody was absent (Fig. 2B).

**DISCUSSION**

To the best of our knowledge, this is the first report in the English literature of the successful application of immunohistochemical techniques to support a diagnosis of LGMD 2A and first report ever using calp3d/12A2 antibodies. One of our patients had convincing evidence of LGMD 2A, including a characteristic phenotype, and concordant diagnostic support from Western blot, immunohistochemistry, and mutational analysis.

There is one previous case report written in Japanese with successful application of immunostaining with monoclonal antibodies against human calpain-3 (2C4).\(^9\) This related to a 45-year-old woman who presented with progressive limb-girdle weakness from age 25 years. In this patient’s muscle biopsy, calpain-3 was completely absent in the sarcoplasm with the ABC method of immunohistochemical staining. However, granular and striated patterns were noted in the control muscle. Diagnosis was confirmed by the absence of the 94 kDa band in Western blot and a homozygotic mutation (Leu 189 Val) in the calpain gene. However, this group relied on the 2C4 antibody for staining. We are the first to demonstrate that the 12A2 antibody could be used reliably as well.

In recent years, Keira et al. used their own rabbit-derived polyclonal antibody to demonstrate the calpain-3 localization in myofibrils and myonuclei *in vivo*.\(^{12}\) The commercially available antibodies substantiated the findings in Western blot but not on the human cryosections. Another group utilized immunofluorescence microscopy with rabbit polyclonal antibodies to identify the myofibril compartments where calpain-1 is concentrated.\(^{13}\) However, the substrate tissue here was postmortem bovine skeletal red muscles. Thus, immunobased techniques have the advantage of facilitating calpain localization within cells.

In contrast, it has been generally accepted that Western blot is the most appropriate method to screen for LGMD 2A. In fact, it has been cited that the limitation for the widespread use of immunodiagnosis for calpain-3 is that the current 2C4 and 12A2 antibodies only work on Western blots and not on tissue sections.\(^{7,14}\)

Nevertheless, Western blot has a number of technical limitations. For instance, in the case of calpain reduction, variability in timing of muscle handling and processing may subject the tissue to autolysis and protein degradation.\(^{15,16}\) This would not be expected to be so much of an issue with immunohistochemistry, though so far such studies have not been completed.

On a practical clinical basis, not all pathology labs have access to Western blotting. In those centers already using immunohistochemical staining for screening of the limb-girdle muscular dystrophies, calpain-3 could be readily added to the existing panel of antibodies. Furthermore, conventional immunoblot protocols require the solubilization of a significant portion of a muscle biopsy specimen (20–30 mg).\(^{17}\) Some institutions may not have sufficient muscle tissue left apart from what is already mounted on the slides and frozen. Immunohistochemistry would be a feasible alternative to screen for calpain-3 deficiency under these circumstances.

Diagnosing calpainopathy on the basis of Western blot, compared with mutational gene analysis, is not sensitive.
Many studies have shown that there is no direct correlation between the amount of calpain-3 detected on Western blots and the severity of the phenotype. A normal amount of CAPN3 on Western blotting may be found in genetically proven calpainopathies, whereas calpain may be reduced in amount or absent in other forms of LGMD as a secondary effect. At this point in time, it is not known how immunohistochemical staining would be affected in secondary reductions of calpain, as seen in dysferlinopathy, fukutin-related protein limb-girdle dystrophy and titinopathy. This would warrant further study, including confirmation by mutation analysis.

In one patient we found the 550 del A mutation in one allele. The veracity of the finding is supported by the 550 del A mutation being a frequent one in calpainopathy.

Furthermore, the detection of only one mutation is relatively common, occurring in about 10–43% of patients with LGMD 2A. This 550delA mutation, at the exon 4, is a frameshift mutation which results in a premature stop codon. If homozygous, a total loss of protein would be expected on Western blot. In heterozygous cases, the final effect on the protein cannot be reliably predicted. This again underscores the importance of other techniques, such as immunohistochemistry, reported here, in confirming the definite diagnosis in patients where the mutation in the second allele cannot be found.

The identification of a protein defect on muscle biopsy is extremely important to select candidate patients for subsequent mutation detection analysis. Mutation screening of the CAPN3 gene is labor-intensive, expensive and time-consuming. We propose that immunohistochemical techniques with commercial calpain antibodies offer a feasible, straightforward and reasonably specific screening alternative to Western blot analysis.

ACKNOWLEDGMENTS

This work was primarily funded by the Hospital for Sick Children Foundation. We also wish to thank Dr Ayako Ochi for translating the Japanese paper published in Rinsho Shinkeigaku.

REFERENCES


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