Atrophy, Fibrosis, and Increased PAX7-Positive Cells in Pharyngeal Muscles of Oculopharyngeal Muscular Dystrophy Patients

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Abstract
Oculopharyngeal muscular dystrophy (OPMD) is a late-onset autosomal dominant inherited dystrophy caused by an abnormal trinucleotide repeat expansion in the poly(A)-binding-protein-nuclear 1 (PABPN1) gene. Primary muscular targets of OPMD are the eyelid elevator and pharyngeal muscles, including the cricopharyngeal muscle (CPM), the progressive involution of which leads to ptosis and dysphagia, respectively. To understand the consequences of PABPN1 polyalanine expansion in OPMD, we studied muscle biopsies from 14 OPMD patients, 3 inclusion body myositis patients, and 9 healthy controls. In OPMD patient CPM (n = 6), there were typical dystrophic features with extensive endomysial fibrosis and marked atrophy of myosin heavy-chain Ila fibers. There were more PAX7-positive cells in all CPM versus other muscles (n = 5, control; n = 3, inclusion body myositis), and they were more numerous in OPMD CPM versus control normal CPM without any sign of muscle regeneration. Intranuclear inclusions were present in all OPMD muscles but unaffected OPMD patient muscles (i.e. sternocleidomastoid, quadriceps, or deltoid; n = 14) did not show evidence of fibrosis, atrophy, or increased PAX7-positive cell numbers. These results suggest that the specific involvement of CPM in OPMD might be caused by failure of the regenerative response with dysfunction of PAX7-positive cells and exacerbated fibrosis that does not correlate with the presence of PABPN1 inclusions.

Key Words: Atrophy, Fibrosis, Intranuclear inclusion, Oculopharyngeal muscular dystrophy, PABPN1, Pharyngeal muscle, Satellite cells.

INTRODUCTION
Oculopharyngeal muscular dystrophy (OPMD) is a late-onset autosomal dominant inherited genetic neuromuscular disease mainly characterized by weakness of the eyelid elevator and pharyngeal muscles, including cricopharyngeal muscles (CPM), leading to ptosis and dysphagia, respectively. Proximal lower limb muscles can also be affected at later stages. Oculopharyngeal muscular dystrophy is caused by an abnormal (GCN) triplet expansion within the poly(A)-binding-protein-nuclear 1 (PABPN1) gene located on chromosome 14 (14q11.2-q13). The wild-type PABPN1 gene contains 10 (GCN) repeats, but the mutated form in OPMD is expanded to 12–17 repeats, adding 2 to 7 additional alanine residues at the N-terminus of the PABPN1 protein (1). The hallmark of OPMD is the presence of mutated PABPN1 aggregated in the form of insoluble filamentous intranuclear inclusions (INIs) in skeletal muscle fibers (2). A few studies have reported abnormalities in muscle histology of CPM from healthy donors (3, 4), but very little is known concerning morphologic changes of this muscle in OPMD (5). The CPM is the main muscle of the upper esophageal sphincter (UES). The UES is located between the pharynx and the esophagus and is usually contracted. Closing the UES prevents air from entering the digestive tract during inspiration, thereby protecting the pharyngolarynx from acid gastric reflux and opening during swallowing (6). The precise mechanism by which PABPN1 polyalanine expansion primarily affects this specific muscle in OPMD is still unknown.

To identify pathologic features that might suggest an underlying mechanism for this selective muscular involvement in OPMD, we performed a detailed histologic and immunohistochemical study of OPMD CPM versus control patient CPM. Controls included inclusion body myositis (IBM) as pathologic controls and clinically unaffected OPMD muscle biopsies.

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MATERIALS AND METHODS

Patients

We analyzed 3 groups of muscle biopsies from 14 OPMD patients, 9 healthy individuals, and 3 IBM patients as follows: 1) 6 affected CPM biopsies from OPMD patients, 4 from controls, and 3 from IBM; 2) 8 unaffected sternocleidomastoid muscle (SCM) biopsies from OPMD patients and 4 from controls; and 3) 5 unaffected quadriceps or deltoid muscle (QM/DTM) biopsies from OPMD patients and 4 from controls (Table). All patients were aged between 44 and 91 years at the time of muscle biopsy, and groups were age and sex matched. Oculopharyngeal muscular dystrophy patients showed a typical clinical phenotype and PABPN1 mutation confirmed by genetic studies. All biopsies were obtained during surgical procedures: OPMD and IBM CPM biopsies were

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Regarding rimmed vacuoles: ++++, more than 4 fibers per section; +++, 3 fibers per section; +, 1 to 2 fibers per section; −, 0 to 1 fiber per section; nd, no rimmed vacuoles.

DTM, deltoid muscle; F, female; IBM, inclusion body myositis; M, male; nd, not determined; OPMD, oculopharyngeal muscular dystrophy; QM, quadriceps muscle; SCM, sternocleidomastoid muscle.
obtained after cricopharyngeal myotomy (7); control CPM were obtained during otolaryngological surgery after informed consent in accordance with the French legislation on ethical rules.

Muscle Histology
Muscle biopsies were mounted on a cryostat and snap frozen in liquid N2-cooled isopentane. Staining was carried out on transverse serial cryosections of muscles (5 μm). For the assessment of tissue morphology and visualization of fibrosis and connective tissue, muscle sections were stained with hematoxylin and eosin and Sirius red for light microscopic examination. Changes in fiber architecture and structural abnormalities were assessed by Gomori trichrome staining. Intracellular lipids were detected using Oil Red O staining.

Electron Microscopy
Muscle biopsies were fixed in 2% paraformaldehyde + 2% glutaraldehyde diluted in 0.1 mol/L phosphate buffer, pH 7.4. After 2% OsO4 postfixation, they were gradually dehydrated in acetone and embedded in Epon resin (EMS, Fort Washington, PA). Ultrathin sections were stained with uranyl and lead citrate and examined with a Philips CM120 electron microscope connected to an SIS Morada digital camera.

Quantification of Myofiber Sizes
Fiber sizes on muscle sections and atrophy and hypertrophy factors were calculated using the method described by Brooke and Engel (8). Briefly, the smaller diameter of muscle fibers was measured for a total of at least 100 fibers, and a histogram of the diameters was plotted. The mean fiber diameter and SD were calculated, and variability coefficients were calculated as follows: SD × 1000/mean fiber diameter. A summary of morphologic findings for each patient is shown in the Table.

Immunofluorescence
Immunostaining were performed on 5-μm-thick cryostat muscle sections fixed in 4% paraformaldehyde. The following primaries antibodies were used: monoclonal anti-PAX7 (DSHB), anti-myosin heavy chain I (MyHC-I), anti-MyHC-IIa, (clone BAD5, clone SC71, respectively; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), monoclonal anti-lamin A/C, anti-spectrin (NCL-LAM A/C clone 636, NCL-Spec1 clone RBC2/3D5; Novocastra, Newcastle-upon-Tyne, UK), and polyclonal anti-laminin (Z0097; Dako, Trappes, France). Detection of immune complexes was performed using the appropriate Alexa Fluor 555 goat anti-mouse, Alexa Fluor 488 goat anti-mouse, or Alexa Fluor 647 goat anti-rabbit (Life Technologies, Grand Island, NY).

For immunodetection of PABPN1 inclusions, sections were preincubated in 1 mol/L KCl solution for 1 hour to remove soluble proteins before incubation with rabbit polyclonal anti-PABPN1 (a gift from Prof. Elmar Wahle, Halle, Germany) and anti-dystrophin antibody (NCL-Dys1, Novocastra). Sections were further incubated with appropriate secondary antibodies and stained with Hoechst (Sigma-Aldrich, St. Louis, MO) to visualize nuclei.

Image Acquisition and Analysis
Images were visualized using an Olympus BX60 microscope (Olympus Optical, Hamburg, Germany), digitized using a CCD camera (Photometrics CoolSNAP fx; Roper Scientific, Tucson, AZ), and analyzed using MetaView image analysis system (Universal Imaging, Downingtown, PA), MetaMorph imaging system (Roper Scientific) software, and ImageJ 1.44o (http://imagej.nih.gov/ij).

RESULTS
Muscle Histology in OPMD Patients
In hematoxylin and eosin stains, the CPMs of all OPMD, control, and IBM patients had internal myonuclei, split fibers, and a large amount of endomysial connective tissue (Fig. 1A). Sirius red staining revealed the presence of approximately 20% of fibrotic tissue in control and IBM CPM biopsies and more than 40% in OPMD CPM (Fig. 1B). Ragged red fibers and rimmed vacuoles were also present in OPMD CPM (data not shown). Moreover, many hypertrophic fibers in OPMD CPM were clearly evident by simple inspection. The histogram of quantification of fiber size changes revealed that most of the muscle fibers were from 5 to 55 μm in OPMD CPM, 5 to 75 μm in control CPM, and 5 to 85 μm in IBM CPM, with mean fiber diameters of 23, 32, and 40 μm, respectively (Fig. 1C, Figure, Supplemental Digital Content 1, part A, http://links.lww.com/NEN/A431, Table). Measurement of the atrophy factor confirmed the presence of many hypertrophic fibers in all CPM compared with normal adult muscles (8), and this number was even higher in OPMD versus control and IBM CPM (Supplemental Digital Content 1, part B, http://links.lww.com/NEN/A431). There was also an increased variability coefficient of fiber size in OPMD CPM biopsies, indicating that OPMD CPM fibers were more atrophied than control and IBM CPM fibers (Supplemental Digital Content 1, part C, http://links.lww.com/NEN/A431).

By immunostaining with the different anti-MHCs, we confirmed that the CPM is predominantly composed of oxidative slow MyHC-I, with a smaller percentage of fast MyHC-IIa fibers (Fig. 2A). We further investigated whether the distribution and fiber size of the muscle fiber subtypes MyHC-I and MyHC-IIa were modified in OPMD CPM; we observed marked atrophy of fast oxidative MyHC-IIa fibers with a slight but not significant reduction in their number (Fig. 2A, B). Myosin heavy chain I fibers were also atrophied in OPMD CPM compared with those in control but to a much lesser extent than MyHC-IIa (Fig. 2C). Neither signs of muscle regeneration, as detected by MyHC neonatal staining, nor inflammation, as detected by CD4 and CD8 immunostaining, were ever seen in OPMD CPM (data not shown).

Hematoxylin and eosin staining of clinically unaffected OPMD muscles, that is, SCM, DTM, and QM, showed slight myopathic features such as internalization of myonuclei and variability in fiber size in OPMD patients; hypertrophic fibers were prominent in QM and DTM (Fig. 3A, Supplemental Digital Content 1, part D, http://links.lww.com/NEN/A431), but no evidence of fiber atrophy was seen (Fig. 3B, Supplemental Digital Content 1, part B, http://links.lww.com/NEN/A431).
Rimmed vacuoles and ragged red fibers, as shown by Gomori trichrome staining, were consistently observed in OPMD QM and DTM (Fig. 3C; Table), but unaffected OPMD muscles did not show increased fibrotic tissue versus control muscles (Fig. 3D).

PAX7-Positive Cells in CPM

Satellite cell number was determined by counting the number of PAX7-positive nuclei localized at the periphery of the muscle fiber and beneath the basal lamina (Fig. 4A–C). The number of satellite cells was calculated as a proportion relative to all nuclei present inside the basal lamina of the muscle fibers. As shown in Figure 4D, the number of satellite cells was dramatically increased in control CPM compared with control SCM and QM (6% vs 2.5%, respectively). In OPMD CPM, this number was even higher (reaching 9%) with a 1.4-fold increase versus control CPM (Fig. 4E). The proportion of satellite cells in clinically unaffected muscles was not different in OPMD versus control subjects (Table). Surprisingly, a significant proportion of PAX7-positive cells were also found in the interstitial space in CPM (3.1% in OPMD, 0.3% and 1% in control and IBM, respectively). When these PAX7-positive cells located in the interstitial space were included in the quantification, the total number of PAX7-positive cells (i.e., ~7%–8% in control and IBM CPM) reached 12% in OPMD CPM (Fig. 4E). The PAX7-positive interstitial cells were never detected in clinically unaffected OPMD and control muscle.

Intranuclear Inclusions

The numbers of nuclei containing PABPN1 INIs were determined by immunohistochemistry after KCl treatment (Fig. 5A). Proportions of INIs ranged from 2% to 16% in OPMD muscles, that is, from 2% to 10% in CPM, from 6% to 16% in SCM, and from 5% to 14% in QM/DTM (Fig. 5B), confirming that INIs are found both in affected and clinically unaffected muscles in OPMD patients. By electron microscopy, we confirmed the typical tubulo-filamentous inclusions (Fig. 5C). We further performed coimmunostaining of myosin heavy chains MyHC-I and MyHC-IIa, together with the PABPN1 staining on SCM, QM, and DTM from OPMD patients (Fig. 5D). We observed no difference in the proportion

FIGURE 1. Morphology of cricopharyngeal muscle (CPM) biopsies. (A) Hematoxylin and eosin stains of CPM from oculopharyngeal muscular dystrophy (opmd), control (ctrl), and inclusion body myositis (IBM) patients. A large amount of connective tissue, hypertrophy of muscle fibers, and internal nuclei are seen in all CPM biopsies. Scale bar = 50 μm. (B) Sirius red analysis of fibrotic tissue in CPM biopsies. Histograms indicate the amount of fibrotic tissue. Control and IBM CPM biopsies (white and light gray bars, respectively) show 20% fibrosis; opmd CPM (gray bar) have a fibrotic substitution value of 40% (** p < 0.01 opmd vs control and vs IBM). (C) Quantification of fiber sizes. Plots of the frequency of fiber diameter show that control CPM (dashed line), opmd CPM (continuous line), and IBM CPM (gray line) are composed of hypertrophic muscle fibers, with a shift toward smaller fibers for opmd CPM. Plotted lines represent the mean of 3 to 6 different muscles for each group; χ² analysis shows control versus opmd CPM, *** p < 0.001; control versus IBM CPM, not significant; opmd versus IBM, *** p < 0.001.
of INIs in each fiber type (0.21 ± 0.05 INI per MyHC-I fiber vs 0.25 ± 0.11 INI per MyHC-IIa fiber; Student t-test, p = 0.5255).

**DISCUSSION**

The principal aim of this study was to compare for the first time affected OPMD CPM and control CPM in a range of individuals of different ages. In addition, these muscles were compared with clinically unaffected muscles from OPMD patients and normal subjects and with CPM in IBM. Inclusion body myositis is the most common acquired muscle disease in older subjects (9), and dysphagia and CPM functional dysfunction have previously been reported in IBM (10, 11). Although IBM and OPMD are different neuromuscular diseases, that is, sporadic and genetic, respectively, both myopathies have common histopathologic end points, such as muscle degeneration, the presence of rimmed vacuoles, and immunopositivity for the protein TDP-43 (12, 13). Cricopharyngeal muscles, along with other pharyngeal and eyelid muscles, are the main targets of the PABPN1 mutation in OPMD, which is associated with slowly progressive dysphagia and bilateral ptosis in the sixth to seventh decade of life. Later, muscle weakness of proximal lower limbs is often observed, but involvement of other body systems is rare (14–16). Dysphagia can occur in adult patients associated with aging (17), several neurologic or neuromuscular disorders (18–21), and with head and neck cancer or head injury (22) and it can lead to death caused by malnutrition, aspiration, or pneumonia. The role of the CPM is to maintain a constant basal tonus and then rapidly relax during swallowing, thereby opening the UES. Its dysfunction in OPMD patients results in impaired swallowing, as evidenced by decreased intraoral swallowing pressures and capacity (23, 24). The CPM is a skeletal muscle that differs morphologically from the limb muscles. Previous histochemical studies have shown that human CPM from healthy donors contains atypical features compared with QM (25). We confirmed that control CPM shows some features that are usually considered to be pathologic in other muscles, that is, hypotrophic muscle fibers surrounded by connective tissue. This could explain, at least in part, the primary and selective impairment of...
pharyngeal and eyelid muscles in OPMD (25–27). However, these atypical features are also present in other normal striated muscle fibers lacking skeletal attachment, such as the human external urethral sphincter muscle (28), which are not clinically affected in OPMD. Indeed, these muscles contain specialized fibers that are capable of maintaining a constant contraction state during prolonged periods, as for UES, where CPM represents the main component (6, 29, 30). In OPMD patient CPM, we found a significant increase in fibrosis compared with normal and IBM CPM that was accompanied by even more pronounced predominance of atrophic muscle fibers. Increased fibrotic tissue was detected in all OPMD patient CPM biopsies with no correlation with age. In contrast, in clinically unaffected OPMD muscles, such as SCM and QM/DTM, no evidence of fibrotic substitution was seen. Fibrotic muscular substitution can be considered a dysregulated tissue

FIGURE 3. Morphologic analysis of unaffected biopsies. (A) Hematoxylin and eosin staining of sternocleidomastoid muscle (SCM) and quadriceps (QM)/deltoid muscle (DTM) biopsies from oculopharyngeal muscular dystrophy (OPMD) and control (CT) patients. There are minor dystrophic features of unaffected OPMD muscle compared with those in cricopharyngeal muscles (CPM). Internal myonuclei and fiber size variation are present in both muscle biopsies. Scale bar = 50 µm. (B) Quantification of fiber sizes. Plots of the frequency of fiber diameter showed no significant fiber diameter difference between OPMD (continuous line) and control (dashed line) unaffected muscle (plotted lines represent the mean of 4 to 5 different muscles for each group). (C) Gomori trichrome staining of OPMD patient unaffected muscle reveals ragged red fibers (asterisk, left panel) and rimmed vacuoles (empty arrow, right panel). Scale bar = 50 µm. (D) Sirius red analysis for quantification of fibrotic tissue. No fibrosis is detected in control muscles (white bars) or unaffected OPMD muscles (gray bars). Data from CPM biopsies are included for comparison.
repair response with severe consequences in neuromuscular disorders, as well as in aging (31), and is attributed to excessive deposition of extracellular matrix components (32). In adult skeletal muscle, the extracellular matrix plays an important role in maintaining muscle structure and providing an appropriate environment for the contractile muscle fibers (33).

A previous analysis of growth factors showed that affected OPMD muscles are characterized by the presence of several growth factors and cytokines that are commonly correlated with the stimulation of fibrosis in liver and lung, further supporting the hypothesis that fibroblast-like cells of muscle tissue could play an important role in the development of fibrosis and dystrophic features in OPMD CPM (34).

Interestingly, the atrophy observed in OPMD CPM was more severe in fast MyHC-IIa than in MyHC-I type fibers. In a previous study, we described such a severe atrophy of fast fibers in muscles of the A17.1 OPMD mouse model (35).

Numerous studies have demonstrated that the loss of skeletal muscle mass related to aging or sarcopenia is characterized by a decline in the number of muscle fibers and specific atrophy of type II fibers (36, 37). We propose that this MyHC fiber–type profile observed in CPM may represent selective premature aging of this muscle in OPMD. Another hypothesis is based on the observation that mitochondrial abnormalities have frequently been described in OPMD patients (38, 39). Because type MyHC-II fibers contain less mitochondria, we postulate that MyHC-II fibers may be more sensitive than MyHC-I to a putative mitochondrial dysfunction occurring in OPMD and thus be primarily affected. This observation emphasizes the potential importance of mitochondrial dysfunction and oxidative stress–related mechanisms that should be studied in more detail in OPMD patient samples.

To understand why there is an increased fibrotic tissue and atrophic muscle fibers without evidence of regeneration...
in affected OPMD muscle, we quantified the proportion of satellite cells using the specific PAX7 marker. Satellite cells represent the main physiologic source of nuclei in the adult that ensure muscle maintenance by myonuclear turnover and fiber repair and regeneration (40). It has been previously demonstrated that, in muscle from young adult subjects, the proportion of satellite cells is approximately 4% to 5%, whereas in muscle from aged subjects, this proportion decreases to approximately 1% (41–43). In our study, the patients ranged from 44 to 91 years old, and we found in SCM, QM and DTM a proportion of satellite cells approximately 2% consistent with values described in aged subjects. However, in CPM biopsies, we detected a drastic increase in the proportion of satellite cells, approximately 6% in controls, reaching more than 10% in OPMD. This increase in OPMD patients is apparently contradictory with an age-dependent phenomenon and did not correlate with age (e.g. 11.6% in the 54-year-old patient vs 10.6% in the 81-year-old patient) (Table). Interestingly, in clinically unaffected OPMD muscles, we did not find any increase in the amount of satellite cells, indicating that this increase in OPMD satellite cells is restricted only to affected muscles, with no signs of ongoing regeneration. In addition, although we never found PAX7-positive cells in the interstitial space in SCM, QM, and DTM, in all CPM, we found a higher proportion of PAX7-positive cells outside muscle fibers, in the interstitial space between fibers, or even in the fibroconnective tissue; this reached significant values in OPMD CPM versus control and IBM CPM. This increase and unusual localization of PAX7-positive cells, together with the presence of atrophic fibers in CPM, could result from 1) defective satellite cell activation process during muscle degeneration or a reduced efficiency of the cross talk between damaged muscle fibers and their precursors; 2) dysfunction of the mechanism of...
asymmetric division; however, asymmetric divisions usually occur during a regenerative process that was not detected in OPMD CPM; 3) a defect in the recognition of the niche, for example, a perturbation of the signals that make satellite cells expressing PAX7 recognize a precise location on the edge of muscle fibers, such as the Notch pathway, whether this defect is on the fiber or on the PAX7-positive cell (44); and/or 4) an abnormal proliferation of these cells, which may be an inefficient attempt to counteract a functional defect. Although there is a difference in the number of PAX7-positive cells in OPMD, it is not clear that the behavior of these cells is abnormal nor is it clear that modification of satellite cell number or behavior contributes to the pathogenesis of OPMD. Further studies will be needed to study the behavior of satellite cells in CPM.

The hallmark of OPMD is the presence of INIs containing mutated PABPN1 in skeletal muscle fibers (2). Poly(A)-binding-protein-nuclear 1 is a ubiquitous protein localized in nuclear speckles that binds with high affinity to poly(A) tails of mRNAs. Poly(A)-binding-protein-nuclear 1 promotes the interaction between the poly(A) polymerase and the cleavage and polyadenylation specificity factor, controls the length of the poly(A) tail (45, 46), suppresses alternative poly(A) sites (47, 48), and contributes to the export of mRNA from the nucleus to the cytoplasm (49, 50). Although PABPN1 is ubiquitously expressed, the clinical and pathologic phenotype is restricted to skeletal muscle in OPMD patients. Whereas several studies have suggested a pathologic function of INIs (51), several other studies have suggested that the INIs might just be the result of a cellular defense mechanism, the soluble form of the protein itself being toxic (52, 53). Using KCl pretreatment followed by PABPN1 immunostaining, we showed that both affected and unaffected muscles of OPMD patients contain INIs, although with a higher number of INIs in unaffected muscles than in affected CPM muscles that are mostly composed of severely atrophied fibers. We did not find any difference in the proportion of INIs in either MyHC-I or MyHC-IIa fibers. Interestingly, by immunostaining, we found a higher proportion of INIs than previously published studies performed using electron microscopy. For example, Tomé et al (54) showed that, of the nuclei seen in every ultrathin section of DTM, 4% to 5% contained INIs, whereas we found a proportion ranging from 2% to 15% in our different samples. This difference is certainly caused by the different techniques used but might indicate that the amount of INIs in OPMD samples has so far been underestimated. The presence of INIs in unaffected muscles where no atrophy or increased satellite cells are observed further emphasizes the complex and poorly understood role of these INIs in OPMD. Our results clearly suggest that INIs are not the only factor involved in muscle atrophy and satellite cell dysfunction. The presence of INIs might be necessary but not sufficient to trigger such effects; atrophy and satellite cell deregulation will only appear once INIs and other factors such as aging, proteasome deficiency, or mitochondrial dysfunction are also in place. Alternatively, INIs may have no link with atrophy, which could be the case if they are related to a defense mechanism.

In conclusion, we showed that OPMD CPM is characterized by increased accumulation of fibrotic tissue, severe atrophy of MyHC-II fibers, and increased number of PAX7-positive cells in both satellite and interstitial positions in the absence of muscle regeneration. In clinically unaffected muscles from OPMD patients, we confirmed the presence of INIs and other pathologic features, such as rimmed vacuoles and ragged red fibers, but these muscles do not show any evidence of fibrosis, atrophy, or increase in satellite cell number. In CPM biopsies from IBM patients, where pharyngeal muscle weakness is present, we also did not observe any increased fibrosis, atrophy, or satellite cell number compared with that in control CPM. Altogether, these results suggest that the specific involvement of CPM in OPMD correlates with a failure of the regenerative response and to an exacerbated fibrosis.

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