Proteomic Analysis of Inclusion Body Myositis

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Abstract

Sporadic inclusion body myositis (IBM) is the most frequently acquired inflammatory myopathy of late adult life, yet its diagnostic criteria and pathogenesis remain poorly defined. Because effective treatment is lacking, research efforts have intensified to identify specific markers for this debilitating disorder. In this study, proteomic analysis of 4 cases of sporadic IBM was compared with 5 cases of inflammatory myopathy without clinicopathologic features of IBM to distinguish the IBM-specific proteome. Proteins were separated by 2-dimensional polyacrylamide gel electrophoresis and profiled by mass spectrometric sequencing. Expression of most proteins remained unchanged; however, 16 proteins were upregulated and 6 proteins were downregulated in IBM compared with cases of non-IBM inflammatory myopathy. These IBM-specific proteins included apolipoprotein A-I, amyloid beta precursor protein, and transthyretin, which have been associated with amyloidosis; superoxide dismutase, enolase, and various molecular chaperones indicate perturbations in detoxification, energy metabolism, and protein folding, respectively. The IBM-downregulated proteins mainly serve as carriers for muscle contraction and other normal muscle functions. We further applied Western blot and immunohistochemistry to verify the proteomic findings. This study validates proteomics as a powerful tool in the study of muscle disease and indicates a unique pattern of protein expression in IBM.

Key Words: Inclusion body myositis, Inflammatory myopathy, Mass spectrometry, Proteomics, 2-D PAGE.

INTRODUCTION

Sporadic inclusion body myositis (IBM) is an indolent, progressive myopathy of older adults that is refractory to therapy. Patients with IBM exhibit a characteristic pattern of clinical involvement consisting of early weakness and atrophy of the quadriceps, volar forearm muscles, and ankle dorsiflexors (1–3). Variable endomysial inflammation that frequently invades nonnecrotic fibers and rimmed vacuoles containing granular material are among the common pathologic findings (1–4). A neurogenic component consisting of angular atrophic fibers either randomly distributed or in small clusters completes the characteristic light microscopic picture. Ultrastructural examination of the vacuoles reveals autophagic debris and 15- to 18-nm tubulofilamentous inclusions (1–3). Recent studies have emphasized the importance of mitochondrial cytopathy in IBM, which can be identified in biopsies by the presence of cytochrome oxidase (COX)-negative fibers (5). Despite the acknowledged clinicopathologic constellation of findings, the pathologic diagnosis is difficult in many patients, in part because of the small number of fibers in frozen sections containing rimmed vacuoles and the difficulty of capturing rimmed vacuoles on plastic-embedded sections for the ultrastructural identification of tubulofilamentous inclusions.

Although of unknown etiology, potential mechanisms underlying this disorder include immune system abnormalities possibly triggered by infectious agents, increased oxidative stress, and muscle aging leading to the accumulation of amyloid beta (Aβ) and other Alzheimer-related proteins (1, 6–16). Because this entity continues to generate considerable controversy, investigators have applied a variety of techniques to clarify the diagnostic features that may yield potential therapeutic targets. Using proteomic analysis, the objective of the present study was to distinguish whether there are proteins uniquely expressed in IBM that will provide candidates for future single protein investigation.

MATERIALS AND METHODS

Patient Characteristic and Pathologic Observations

Muscle biopsy specimens from 4 cases of sporadic IBM and 5 cases of inflammatory myopathy were collected from the files of the Armed Forces Institute of Pathology (AFIP). Table 1 summarizes the clinical characteristics and pathologic findings. Tissue and clinical information were obtained as part of an Institutional Review Board-approved study at the AFIP. We selected 4 cases from over 100 cases of IBM because of their classic clinical history and morphologic features, especially the abundance of rimmed vacuoles. All of the cases were from patients with clinically suspected IBM and none of the cases...
came from patients with a known family history of IBM to exclude the possibility of the inherited form of the disease. The average age for the 3 male and one female patients with IBM was 63 years (range, 48–77 years). Of the 5 cases of non-IBM inflammatory myopathy, there were 2 males and 3 females with an average age of 62 years (range, 57–68 years). These cases were selected because they lacked clinicopathologic criteria suggestive of IBM. Specifically, they lacked rimmed vacuoles, significant neurogenic features, or COX-negative fibers. Histopathologic examination of these cases revealed variable chronic inflammatory infiltrates accompanied by myopathic features (regeneration, degeneration, and myophagocytosis).

Available on all cases were a standard battery of enzyme histochemical preparations performed on frozen tissue that included myoadenylate deaminase, myofibrillar, adenosine triphosphatase at pH 10.4, myofibrillar, adenosine triphosphatase at pH 4.5, nicotinamide adenine dinucleotide–tetrazolium reductase, alkaline phosphatase, nonspecific esterase, and immunohistochemical methods for staining the major histocompatibility complex class 1 (MHC-1). Histologic methods that included hematoxylin and eosin (H&E), periodic Schiff, and modified Gomori trichrome were performed on paraffin-embedded tissue. Mitochondrial stains, COX, and succinate dehydrogenase performed on frozen tissue were also available on all cases. Electron micrographs were available for all IBM cases.

The histopathologic diagnosis of IBM was based on criteria promulgated by Griggs et al (3). In all 4 cases of IBM, there was variable chronic inflammatory cell infiltration with invasion of nonnecrotic muscle fibers, abundant rimmed vacuoles highlighted by the Gomori trichrome stain, and a neurogenic component characterized by scattered esterase-positive, angular atrophic fibers, and occasional pyknotic nuclear clumps (Fig. 1A–C). Other myopathic features consisted of scattered regenerating/degenerating fibers and myophagocytosis. Although the inflammatory component was mild in 2 cases, MHC-1 was diffusely unregulated in all cases (Fig. 1E). Two cases revealed more extensive endomysial fibrosis. COX-negative fibers were present in 3 cases (Fig. 1D); no significant changes were noted on succinate dehydrogenase-stained sections. In all cases, electron microscopy revealed intracytoplasmic inclusions consisting of 15- to 18-nm filaments. The histopathologic diagnosis of IBM was based on criteria promulgated by Griggs et al (3). In all 4 cases of IBM, there was variable chronic inflammatory cell infiltration with invasion of nonnecrotic muscle fibers, abundant rimmed vacuoles highlighted by the Gomori trichrome stain, and a neurogenic component characterized by scattered esterase-positive, angular atrophic fibers, and occasional pyknotic nuclear clumps (Fig. 1A–C). Other myopathic features consisted of scattered regenerating/degenerating fibers and myophagocytosis. Although the inflammatory component was mild in 2 cases, MHC-1 was diffusely unregulated in all cases (Fig. 1E). Two cases revealed more extensive endomysial fibrosis. COX-negative fibers were present in 3 cases (Fig. 1D); no significant changes were noted on succinate dehydrogenase-stained sections. In all cases, electron microscopy revealed intracytoplasmic inclusions consisting of 15- to 18-nm filaments. In all cases, electron microscopy revealed intracytoplasmic inclusions consisting of 15- to 18-nm filaments. In all cases, electron microscopy revealed intracytoplasmic inclusions consisting of 15- to 18-nm filaments.
a Multiphor II Flated System (Amersham Biosciences) under a constant voltage of 700 V for 3 hours. A silver staining kit (Amersham Biosciences) was used according to the manufacturer’s instructions to detect protein spots. All samples were run in duplicate to guarantee over 90% identity (see B Image Analysis) before performing further experiment.

Image Analysis and Gel Digestion

The 2-D PAGE images from all 9 specimens were assigned into IBM (n = 4) and non-IBM (n = 5) groups before being analyzed. The intensities of protein spots on each 2-D gel were analyzed with Proteomweaver according to the manufacturer’s instructions to detect protein spots. All samples were run in duplicate to guarantee over 90% identity (see “Image Analysis”) before performing further experiment.

RESULTS

Duplicated 2-D PAGE, performed in a manner blinded to the tissue diagnosis, achieved a consistent proteomic pattern of each sample (supplemental figure). According to the computerized comparison between 2 groups (4 images of the IBM group with 5 images of the non-IBM group) differentially expressed protein spots were marked onto the representative 2-D PAGE images (case 1 in the IBM group and case 1 in the non-IBM group; Fig. 2). Tandem mass spectrometry sequencing was performed on these discrete protein spots and a total of 22 proteins (approximately 900 protein spots were recognized from each gel) were found to distinguish IBM from non-IBM inflammatory muscle (16 in Table 2 upregulated; A–E in Table 3: downregulated in IBM).

Contractile Proteins

In striated muscle, force is generated by conversion of chemical energy in the form of ATP into mechanical energy. This process is mediated through the interaction among myosin, the major protein of thick filaments, and actin, the...
principal thin filament (20). Most of the proteins that showed decreased expression in IBM are contractile-related. These include different forms of myosin (A, D, and F in Table 3) and troponin (B and C in Table 3). The latter group is an important component of the skeletal muscle contractile apparatus. The alpha 1 actin precursor (5 in Table 2) and the fast skeletal myosin alkali light chain (14 in Table 2) are upregulated in IBM tissue, indicating the differential dysfunction of muscle contraction between the 2 myopathies.

**Protein Unfolding or Misfolding**

Evidence of protein unfolding or misfolding in IBM was provided by the selective upregulation of various molecular chaperones, including heat shock protein (Hsp) 27 (spots 6, 12, and 15), Hsp 20 (spot 11), and DJ-1 (spot 10) (21).

**Energy Metabolism**

Proteins involved in energy metabolism include a number of glycolytic and oxidative phosphorylation enzymes. Members of the enolase family catalyze the dehydration of 2-phosphoglycerate to phosphoenolpyruvate (24). Compared with non-IBM inflammatory muscle, IBM expresses a higher level of enolase 1 (spot 2) and muscle specific beta-enolase (spot 4). Another protein indicating perturbations of energy metabolism in IBM was the upregulated creatine kinase (spot 3) (21–23).

**Detoxification of Cytotoxic Products**

Cellular superoxide anion (O$_2^-$) is a normal byproduct of the mitochondrial electron transport chain. Superoxide dismutase (SOD) catalyzes the dismutation of O$_2^-$ yielding relatively stable hydrogen peroxide (H$_2$O$_2$) (29). SOD (spot 13) is sharply increased in IBM tissue. Peroxiredoxins (spots 7 and 9) are involved in the redox regulation of the cell, eliminating peroxides generated during metabolism through the thioredoxin system (30).

**Amyloidosis-Related Proteins**

An important observation related to the pathogenesis of IBM is the upregulation of transthyretin (spot 16) (31). Defects in transthyretin are among the causes of certain types of amyloidosis, complementing data that suggests IBM is a degenerative muscle disorder. Amyloid beta A4 protein precursor (AβPP, spot 1) and apolipoprotein A-I preproprotein (APOA1, spot 8) are closely involved in amyloidosis (32, 33).

Subject to the availability of antibodies, we performed Western blot and immunohistochemistry on selected proteins that were identified from MS/MS sequencing. We compared Apo A–I, Hsp27, and DJ-1 expression in 4 IBM tissues with that in 5 non-IBM inflammatory muscle tissues. Western blotting quantitatively indicated overexpression of these proteins (Fig. 3A). Immunohistochemical staining detected diffuse positive signal (light brown) within the sarcolemma with greater intensity (dark brown) in the rimmed vacuoles (Fig. 3B).

**DISCUSSION**

The term IBM was coined by Yunis and Samaha in 1971 to describe a case of chronic polymyositis that showed distinctive cytoplasmic rimmed vacuoles, eosinophilic inclusions in the cytoplasm, and rare intranuclear inclusions (34). Sporadic and inherited cases encompass the spectrum of this disease, leading some authors to postulate that the morphologic changes may represent a common histologic end point resulting from different etiologies. In sporadic IBM, 2 key pathogenetic hypotheses (which appear to be interrelated) are immune dysregulation and an amyloid-related degenerative process (2, 6). Detection of rimmed vacuoles by light microscopy combined with ultrastructural demonstration of tubulofilaments is still the gold standard for the routine diagnosis of IBM, but provides little insight into the constituent elements or specificity of the hallmark inclusions.

Myositis is a multitiered process with numerous, interdependent interactions among a variety of gene products.
Among the most versatile of the genomic techniques are DNA microarrays, also known as DNA microarrays or oligonucleotide high-density arrays of oligonucleotides or complementary DNAs, also known as DNA microarrays or oligonucleotide arrays. Greenberg et al reported that various subtypes of inflammatory myopathies have distinct gene expression signatures (35). In another study, the authors detected unique inflammatory myopathies have distinct gene expression patterns of gene expression in patients with dermatomyositis compared with the single candidate approach as a result of its immense statistical power that has the potential to discover pathways not detected by other methods. Despite the fact that microarray data are based on hybridization, an indirect tool that reveals gene expression, it can only represent the mRNA expression level. With recent improvements in proteomic techniques, proteome profiling has become a powerful complementary approach to nucleic acid-based molecular profiling in large-scale gene analyses. Mass spectrometry—proteomic profiling has emerged as an equally formidable platform to identify the components of small protein complexes, protein interactions, or determine posttranslational modifications and biomarkers. Basically, this technique catalogs proteins based on a combination of methods that include 2-D PAGE or other non-gel-based protein separation tools, MS/MS analysis of molecular mass, and sequencing of electrophoretically divided proteinaceous material with subsequent analysis using bioinformatic methods. 2-D PAGE-based proteomic profiling, as the only quantitative tool for

### TABLE 2. Upregulated Proteins in Sporadic Inclusion Body Myositis Compared With Inflammatory Muscle Tissues

| Protein Name | ID       | Gene Locus | Potential Function |
|--------------|----------|------------|--------------------|
| 1. Amyloid beta A4 protein precursor (ABPP) | gi|4502167 | 21q21.2 | Forms the protein basis of the amyloid plaques found in the brains of patients with Alzheimer disease |
| 2. Enolase 1 | gi|4503571 | 1p36.3 | Multifunctional enzyme in glycolysis, growth control, hypoxia tolerance, and allergic responses; also serves as an activator of plasminogen on the cell surface leukocytes and neurons and participate in the intravascular and pericellular fibrinolytic process |
| 3. Muscle creatine kinase | gi|21536288 | 19q13.2 | Reversibly catalyzes the transfer of phosphate between ATP and various phosphagens (e.g. creatine phosphate); creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands such as skeletal muscle, heart, brain, and spermatozoa |
| 4. Enolase 3, beta, muscle | gi|16554592 | 17p11 | Involved in striated muscle development and regeneration through catalyzing 2-phospho-D-glycerate into phosphoenolpyruvate |
| 5. Alpha 1 actin precursor | gi|4501881 | 1q42.13 | Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells |
| 6. Heat shock 27-kDa protein | gi|450451 | 7q11.23 | Involved in stress resistance and actin organization |
| 7. Peroxiredoxin 6 | gi|4758638 | 1q25.1 | Involved in redox regulation, phospholipids turnover, and protection against oxidative injury |
| 8. Apolipoprotein A–I preproprotein (Apo A–I) | gi|4557321 | 11q23 | Participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase (LCAT) |
| 9. Peroxiredoxin 2 isoform a | gi|32189392 | 19p13.2 | Involved in redox regulation and peroxides eliminating; reduces peroxides with reducing equivalents provided through the thioredoxin system; participates in the signaling cascades of tumor necrosis factor-alpha |
| 10. DJ-1 protein | gi|31543380 | 1p36.33 | Acts as positive regulator of androgen receptor-dependent transcription; function as redox-sensitive chaperone and as sensor for oxidative stress; protects neurons against oxidative stress and cell death |
| 11. Heat shock protein, alpha-crystallin-related, B6 | gi|21389433 | 19q13.12 | Belongs to the small heat shock protein (hsp20) family; plays a vital role in embryonic development |
| 12. Heat shock 27-kDa protein family, member 7 | gi|7657202 | 1p36.23 | Belongs to the small heat shock protein family; the synthesis of Hsp27 is induced by heat shock and other environmental and pathophysiological stresses such as ultraviolet radiation, hypoxia, and ischemia; Hsp27 may act as energy-independent traps preventing irreversible protein aggregation under stress |
| 13. Superoxide dismutase 1 | gi|4507149 | 21q22.1 | Destroys radicals by catalyzing 2 superoxide into hydrogen peroxide |
| 14. Fast skeletal myosin alkali light chain 1 isoform 1f | gi|17986273 | 2q33 | Regulatory light chain of myosin; does not bind calcium |
| 15. Heat shock 27-kDa protein family, member 7 | gi|7657202 | 1p36.23 | Same as 17th protein |
| 16. Transthyretin | gi|4507725 | 18q12.1 | Thyroid hormone-binding protein; involved in thyroxine transport; defects in transthyretin cause amyloidosis |

Given this diversity, molecular profiling may have advantages compared with the single candidate approach as a result of its ability to interrogate multiple genes and their products. Among the most versatile of the genomic techniques are high-density arrays of oligonucleotides or complementary DNAs, also known as DNA microarrays or oligonucleotide arrays. Greenberg et al reported that various subtypes of inflammatory myopathies have distinct gene expression signatures (35). In another study, the authors detected unique patterns of gene expression in patients with dermatomyositis who responded to therapy with intravenous immunoglobulin and are ubiquitously expressed in all eukaryotic cells.
molecular profiling, is well suited to discovering unexpected changes during disease progression or therapeutic intervention. The present study represents the first attempt to generate a comprehensive protein database that may provide another valuable resource for the study of IBM and other muscle diseases.

Of the 16 differentially expressed proteins in the present IBM study, we identified apolipoprotein A-I, a major component of high-density lipoproteins that is also one of the major amyloid fibril proteins and a minor constituent of senile plaques in Alzheimer disease (1, 6–10, 13, 15, 16, 38, 39). Several lines of evidence suggest that oxidative stress in aging muscle combined with inflammation leads to the formation of intracellular amyloid in IBM. Dalakas reported that interleukin-1, secreted by the chronically activated endomysial inflammatory cells, upregulates AβPP gene expression and AβPP promoter and co-localizes with AβPP within the vacuolated muscle fiber (2).

### TABLE 3. Upregulated Proteins in Inflammatory Muscle Tissues Compared With Sporadic Inclusion Body Myositis

| Proteins | Protein Name | ID          | Gene Locus | Potential Function                                           |
|----------|--------------|-------------|------------|-------------------------------------------------------------|
| A        | Myosin heavy chain 6 | gi|27764861   | 14q11.2 | Muscle contraction                                           |
| B        | Tropomyosin 2 (beta) isofrom 1 | gi|42476296   | 9p13.3  | Binds to actin filaments in muscle cells; plays a central role, in association with the troponin complex, in the calcium-dependent regulation of vertebrate striated muscle contraction |
| C        | Troponin T1, skeletal, slow | gi|39930527   | 19q13.42 | The tropomyosin-binding subunit of troponin, the thin filament regulatory complex, which confers calcium sensitivity to striated muscle actomyosin ATPase activity |
| D        | Myosin, heavy polypeptide 7, cardiac muscle, beta | gi|4557773   | 14q11.2  | Muscle contraction                                           |
| E        | Crystallin, alpha B | gi|4503057   | 11q23.1  | Selectively expressed in slow-twitch oxidative muscle fibers with chaperone-like activity and maintenance of cytoskeletal network in muscle, involved in desmin-related myopathy |
| F        | Myosin light chain 2 | gi|4557775   | 16p11.2  | Involved in the regulation of myosin ATPase activity in smooth muscle |

**FIGURE 3.** Validation of the proteomic results by Western blot and immunohistochemistry. (A) Western blot: selected candidates (3 of 21) from the proteins distinguishing inclusion body myositis (IBM) (lanes 6–9) from non-IBM inflammatory myopathy (lanes 1–5) were applied to Western blot. Compared with non-IBM inflammatory myopathy, Apo A-I (31 kDa), Hsp27 (23 kDa), and DJ-1 (20 kDa) were upregulated in IBM. (B) Immunohistochemistry (original magnification: 400x): the expression of Apo A-I (Ac, Ai), Hsp27 (Hc, Hi), and DJ-1 (Dc, Di) were also investigated by immunohistochemical staining. The left 3 panels indicate non-IBM inflammatory myopathy; the right panels are IBM specimens. All 3 antibodies detected stronger immunosignal (dark brown) in IBM than in non-IBM inflammatory myopathy and the majority staining localized in rimmed vacuoles.
Askanas and Engel hypothesized that the overexpression of AbPP within the aging muscle fibers is an early upstream event causing a subsequent pathogenetic cascade. According to their observations, several processes seem to be important to the pathogenesis of IBM: 1) increased transcription and accumulation of AbPP, accumulation of its proteolytic fragment Abβ; 2) abnormal accumulation of cholesterol, caveolin-1, and apolipoprotein; 3) oxidative stress; 4) accumulations of intramuscular fiber multiprotein aggregates; and 5) unfolded/misfolded proteins (1).

In our study, various molecular chaperones, including Hsp 27 and the not previously described Hsp 20 and DJ-1, were selectively upregulated in IBM cases. Molecular chaperones are quality control agents that are critical for cell viability. Their induction occurs in response to cellular conditions causing unfolded proteins (16, 21, 22, 27, 40). The Hsp70 family of molecular chaperones acts to prevent protein misfolding, imports proteins into organelles, unravels protein aggregates, and enhances cell survival under stress conditions (21). In IBM, Ozturk et al described Hsp70-immunoreactive inclusions that colocalized with Hsp40, Hip, and Bag1, as well as with Abβ and p-tau-immunoreactive inclusions in the majority of vacuolated and several non-vacuolated muscle fibers (41).

Although most of the research on Hsp20 has been on cardiac muscle, recent data suggest that it may modulate actin cytoskeletal dynamics by competing with the actin depolymerizing protein coflin for binding to the scaffolding protein 14-3-3 (42). Little is known about the role of DJ-1 in IBM, but it has been linked to early-onset Parkinson disease, perhaps through disruption of mitochondrial function. In this setting, DJ-1 protects dopaminergic neurons from oxidative stress through upregulation of glutathione synthesis and from the toxic consequences of mutant human alpha-synuclein through increased levels of enolase (21). In IBM, the authors concluded that proteasome dysfunction in IBM muscle fibers may contribute to the accumulation of misfolded, potentially cytotoxic proteins and may be induced by increased intracellular AbPP/Abβ. Mutant ubiquitin, a product of “molecular misreading,” appears to be cytotoxic because its ubiquitinated form inhibits the proteasome and contributes to accumulation of misfolded proteins (misfolded amyloid-β and phosphorylated-tau) and their ensuing toxicity (12). Clusterin is a multiple-function protein that participates in Abeta-amyloid, PrP(res), and α-synuclein aggregation in Alzheimer disease, prionopathies, and α-synucleinopathies, respectively. In IBM and other myofibrillar myopathies, Ferrer et al reported clusterin immunoreactivity in association with abnormal protein deposits (11).

We also isolated transthyretin, which was initially discovered in IBM using immunohistochemical methods (15).

Askanas et al reported a 70-year-old black man who was homozygous for the transthyretin Val122Ile allele and who had both sporadic IBM and cardiac amyloidosis (8, 10). Using cultured muscle fibers from the same patient, the authors observed vacuolation, congophilic inclusions, and clusters of immuno-colocalizing amyloid beta-peptide (Abβ) and transthyretin accumulations (8, 10).

We detected significant perturbations of cellular energy homeostasis characterized by increased levels of enolase and the upregulation of creatine kinase. Oxidative stress may also contribute to mitochondrial dysfunction in IBM (5). Superoxide in the mitochondrial matrix has been proposed to activate uncoupling proteins, thus providing a feedback mechanism that will lower respiratory chain superoxide production by increasing a proton leak across the inner mitochondrial membrane (44). Although the precise role of increased SOD in the setting of IBM is unknown, its induction may function as a protective mechanism against further oxidative stress in the aging milieu (45). Given that denervation atrophy is a common biopsy feature of IBM, it is interesting to note that SOD mutations are associated with familial amyotrophic lateral sclerosis, a denervating condition that can occasionally mimic a myopathy (46).

Although not practical for routine diagnostic work, our observations suggest that proteome technology, which analyzes protein expression profiles, provides a promising new approach to study diseases of skeletal muscle. By focusing on multiple proteins, instead of one, the odds of finding useful disease associations and potential therapeutic targets is enhanced by proteome analysis. In the present study, a unique constellation of protein expression abnormalities were identified in the IBM cases, some of which have not been previously reported. Further studies that include a larger number of cases will be required to establish the precise biologic role of these proteins in IBM. More research of inflammatory muscle diseases using this novel technology may provide the basis for earlier and more accurate diagnosis, prognosis, and therapeutic intervention.

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Supplemental Figure

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