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Diagnostic value of markers of muscle degeneration in sporadic inclusion body myositis

O. Dubourg[1], J. Wanschitz[2], T. Maisonobe[1], A. Béhin[3], Y. Allenbach[4], S. Herson[4], O. Benveniste[4]

[1] Laboratoire de Neuropathologie, Institut de Myologie, Assistance Publique, Hôpitaux de Paris, Hôpital Pitié-Salpêtrière, Université Pierre et Marie Curie, Paris, France; [2] Clinical Department of Neurology, Innsbruck Medical University, Innsbruck, Austria; [3] Centre de Référence des maladies neuromusculaires Paris-Est, Institut de Myologie, Assistance Publique, Hôpitaux de Paris, Hôpital Pitié-Salpêtrière, Université Pierre et Marie Curie, Paris, France; [4] Service de Médecine Interne, Institut de Myologie, Assistance Publique, Hôpitaux de Paris, Hôpital Pitié-Salpêtrière, Université Pierre et Marie Curie, Paris, France

Sporadic inclusion body myositis (s-IBM) is characterized histologically by the association of concomitant inflammatory and degenerative processes. We evaluated the sensitivity and specificity of different markers of the degenerative process in order to refine the histological diagnosis. We performed an immunohistochemical study with antibodies directed against ubiquitin, amyloid-β precursor protein (AβPP), amyloid-β (Aβ), SMI-31, SMI-310, Tar-DNA binding protein-43 (TDP-43) and p62 on s-IBM and control muscle biopsies. Based on conventional stains 36 patients with characteristic clinical features of s-IBM were subclassified as presumed definite s-IBM (*d* s-IBM, n = 17) or possible s-IBM (*p* s-IBM, n = 19) according to the presence or absence of vacuolated muscle fibers. Immunohistochemically, TDP-43 and p62 were the most sensitive markers, accumulating in all *d* s-IBM and in 31% and 37%, respectively, of the *p* s-IBM cases and thus enabling reclassification of these cases as *d* s-IBM. We recommend using TDP-43 and p62 antibodies in the histological diagnosis workup of s-IBM. The specificity of these markers has to be further validated in prospective series.

Key words: inclusion body myositis, inflammatory myopathy, protein aggregate myopathy, immunohistochemistry, Tar-DNA binding protein-43, p62

Introduction

Sporadic inclusion body myositis (s-IBM) is the most common acquired inflammatory myopathy in patients over the age of 50 years (1). It is characterized by slowly progressive, asymmetric atrophy and weakness of both proximal and distal muscles, predominantly affecting the finger and wrist flexors and quadriceps (1). Pathological features include two processes occurring in parallel: one degenerative and one inflammatory. The degenerative process results in atrophic muscle fibers, vacuolar degeneration and accumulation of multiple proteins in vacuolated or non-vacuolated muscle fibers including ubiquitin, amyloid-β precursor protein (AβPP), amyloid-β (Aβ), phosphorylated-tau (p-tau) and many other proteins (2). These protein aggregates form multiple or single foci of amyloid, due to their β-pleated-sheet configuration, present in 60 to 80% of the s-IBM vacuolated muscle fibers, within vacuoles or mostly in the non-vacuolated cytoplasm (3). Phosphorylated tau is visible ultrastructurally as 15-21 nm-diameter paired-helical filaments (PHF) or tubulofilaments and is present in the cytoplasm of vacuolated fibers or rarely in the nuclei (3). The inflammatory process is characterized by endomysial infiltrates of mononuclear cells with CD8+ cytotoxic T-lymphocytes invading major histocompatibility class I-expressing non-necrotic muscle fibers (4).

According to Griggs' criteria (1995), both processes are mandatory for a diagnosis of definite s-IBM (*d* s-IBM). Furthermore, biopsy must show either amyloid deposits evidenced by a fluorescent method or tubulofilaments by electron microscopy. Both methods are not performed routinely everywhere and their sensitivity has never been evaluated. If inflammation only is present, the pathological diagnosis of possible s-IBM (*p* s-IBM) is made. The alternative diagnosis is that case is polymyositis. Differentiating both is important since polymyosi-

Address for correspondence: Odile Dubourg, Laboratoire de Neuropathologie, Assistance Publique, Hôpitaux de Paris, Hôpital Pitié-Salpêtrière, Paris, France. Tel. +0033142161884. Fax +0033142161899. E-mail: odile.dubourg@psl.aphp.fr

tis usually responds to corticosteroids and conventional immunosuppressive treatments, whereas s-IBM does not (5). However, in clinical practice, there is clear evidence that some patients have clinical features of s-IBM, although muscle samples lack the canonical features of s-IBM, even on repeated biopsies (6). This can be attributed either to the patchy distribution or to the late appearance of degenerative features in some patients. In the era of the search for effective treatments of this chronic, progressive and disabling disorder, there is a need for easy and reliable methods to refine the diagnosis of s-IBM (7).

The purpose of this study was to evaluate and compare the sensitivity and specificity of different markers of degeneration accumulated in s-IBM muscles in order to determine if some of them can be recommended in the histological diagnosis workup.

Patients and methods

Patients

Skeletal muscle from 36 patients with s-IBM (19 females, 17 males), were obtained for diagnostic purposes after informed consent at the Reference Center for Neuromuscular Diseases at the Institut de Myologie, Hopital Pitié-Salpêtrière. The assessment of amyloid deposits (by fluorescence-enhanced Congo-red or crystal violet staining) and phosphorylated-tau (by electron microscopy as PHF) on muscle biopsies was not so far routinely performed, not permitting to fulfill *a priori* all Griggs' criteria for *d* s-IBM. Nevertheless, all patients presented clinical and electrophysiological features characteristic of s-IBM (1). On pathological analysis, we distinguished two groups of s-IBM for comparison. We called the first presumed definite s-IBM (*d* s-IBM, n = 17, 8 females, 9 males) and the second possible s-IBM (*p* s-IBM, n = 19, 12 females, 7 males). Presumed *d* s-IBM was defined by the presence of inflammation including invasion of nonnecrotic fibers by mononuclear cells, and vacuoles (rimmed or not). Possible s-IBM was defined by the presence of inflammation with or without invaded fibers but no or single vacuole. Two patients had two biopsies, performed at 2 and 4 years of interval, the first classified as *p* s-IBM and the second as presumed *d* s-IBM. Muscles from patients with polymyositis or dermatomyositis (PM/DM, n = 7), muscular dystrophies (MD, n = 8, including 3 dysferlinopathies, 3 calpainopathies, 1 Bethlem myopathy, 2 unspecified limb girdle muscular dystrophies) and normal muscle biopsies from individuals who underwent diagnostic procedures for myalgia and fatigue (N, n = 6) served as controls. Age at biopsy was similar between patients with presumed *d* s-IBM, *p* s-IBM and controls. It was significantly younger for patients with PM/DM and MD (data not shown). The study was approved by the local ethics committees of the Pitié-Salpêtrière Hospital, Paris, France. Age at biopsy and disease duration did not differ between male and female patients (data not shown).

Methods

Skeletal muscle biopsies were classified as presumed *d* s-IBM and *p* s-IBM according to the abnormalities observed on conventional stains (haematoxilin-eosin and Gomori trichrome). Immunohistochemistry was performed on 8 µm thick cryosections, which were air-dried and fixed either in 4% formaldehyde for Aβ at room temperature or in cold acetone at –20°C (for all other primary antibodies) for 10 minutes each. After washing in phosphate buffered saline (PBS) the sections were incubated with 10% normal goat serum (G9023; Sigma) in antibody diluent (S3022; Dako) for 30 minutes to minimize unspecific binding. Primary antibodies (all mouse monoclonal; except against TDP-43 and p62: rabbit polyclonal) were directed against phosphorylated neurofilament (SMI-31; 1:1000 and SMI-310; 1:1000, Covance), Aβ (β-A4; 1:50; Dako), ubiquitin (1:1000; Dako), AβPP (1:1000, Millipore), TDP-43 (1:2000; Proteintech Group), p62 (1:100; Santa Cruz Biotech). Stainings for Aβ were performed manually with an incubation of the primary antibodies overnight at 4°C. Other stains were done with an automated slide staining system (BenchMark XT, Ventana medical systems). Binding of the primary antibodies was detected with a peroxidase reaction and visualized with 3,3'-diaminobenzidine as chromogen (Dako Real™ Detection System (K5001; Dako) for manual stains; secondary reagents from Ventana medical systems for automated stains). The primary antibody was omitted for control purposes, and IgG1 isotype controls were included in the protocol. The percentages of fibers containing cytoplasmic deposits were determined on serial sections by counting 200 fibers in 20 random fields with a 40x objective.

Results

Percentage of cases with immunoreactive fibers

In order to investigate which marker would turn out most useful for diagnostic purposes, we calculated the percentage of cases with immunoreactive fibers in each category of muscles biopsies (Table 1). None of the normal muscle controls had immunoreactive fibers for the different markers tested. p62 and TDP-43 immunoreactive fibers were observed in all presumed *d* s-IBM cases and in 37 and 31% of *p* s-IBM, respectively. That means that: 1) *a posteriori* all *d* s-IBM patients were well classified and finally, by the presence of PHF evidenced by p62 (Nogalska et al., 2009) fulfill all the Griggs criteria for *d*

Table 1. Percentage of cases with immunoreactive fibers. [1]: one polymyositis, [2]: one Bethlem myopathy, [3]: one polymyositis and one dermatomyositis, [4]: one dysferlinopathy.

	d s-IBM (n = 17)	p s-IBM (n = 19)	PM/DM (n = 7)	Dystrophies (n = 8)	N (n = 6)
p62	100%	37%	14%[1]	0%	0%
TDP43	100%	31%	0%	0%	0%
SMI 31	93%	15%	0%	12.5%[2]	0%
Ubiquitin	87%	31%	28.5%[3]	12.5%[4]	0%
Aβ	13%	0%	0%	0%	0%
APP	13%	0%	0%	0%	0%
SMI 310	13%	0%	0%	0%	0%

s-IBM. The percentage was increased to 42% by adding the cases of *p* s-IBM re-classified with each marker. TDP-43 deposits were not observed in any of the pathological controls. p62 deposits were observed in one case of polymyositis. SMI-31 and ubiquitin aggregates were present in 93% and 87% of presumed *d* s-IBM, and in 15% and 31% of *p* s-IBM, respectively. Ubiquitin aggregates were as frequent in *p* s-IBM as TDP-43 aggregates but also observed in 28.5% of PM/DM muscles and 12.5% of MD muscles. SMI31 aggregates were observed in 12.5% of MD muscles. The other markers were present in 13% of presumed *d* s-IBM but in none with *p* s-IBM.

Percentage of immunoreactive fibers per cases

Table 2 shows the percentage of immunoreactive fibers per cases. The percentage of immunoreactive fibers was similar for TDP-43 and p62 in presumed *d* s-IBM and *p* s-IBM. Immunoreactivity was present either as small multiple granular aggregates in the sarcoplasm of non-vacuolated fibers or larger rounded deposits in the vacuole of the vacuolated fibers. In vacuolated fibers, they could be associated with small multiple granular aggregates in the non-vacuolated cytoplasm (Fig. 1). In cases diagnosed as *p* s-IBM, both markers permitted to detect immunoreactive fibers with the same characteristics as those described before, thus permitting to re-classify some biopsies initially classified with the routine staining as *p* s-IBM in presumed *d* s-IBM. The percentage of immunoreactive fibers per biopsy was much less frequent for the other markers.

Discussion

Sporadic inclusion body myositis is a unique disorder among inflammatory myopathies, characterized pathologically by the presence of mononuclear cell inflammation associated with degenerative features including atrophy

Table 2. Percentage of immunoreactive fibers per biopsy for each marker.

	d s-IBM (n = 17)	p s-IBM (n = 19)	PM/DM (n = 7)	MD (n = 8)	N (n = 6)
p62	12.1 ± 6.5 (1.5-28)	1.9 ± 2.8 (0-8)	1.6 ± 4.1 (0-11)	0	0
TDP43	11.6 ± 11.2 (0.5-47.5)	2.1 ± 3.8 (0-13.8)	0	0	0
SMI 31	3.4 ± 2.5 (0-6.5)	0.05 ± 0.9 (0 – 0.5)	0	0.06 ± 0.2 (0 – 0.5)	0
Ubiquitin	1.1 ± 1.2 (0 – 3.5)	0.1 ± 0.3 (0 – 1)	0.1 ± 0.2 (0 – 0.5)	0.06 ± 0.2 (0 – 0.5)	0
Beta A4	0.1 ± 0.2 (0 – 1)	0	0	0	0
APP	0.1 ± 0.2 (0 – 1)	0	0	0	0
SMI 310	0.1 ± 0.2 (0 – 1)	0	0	0	0

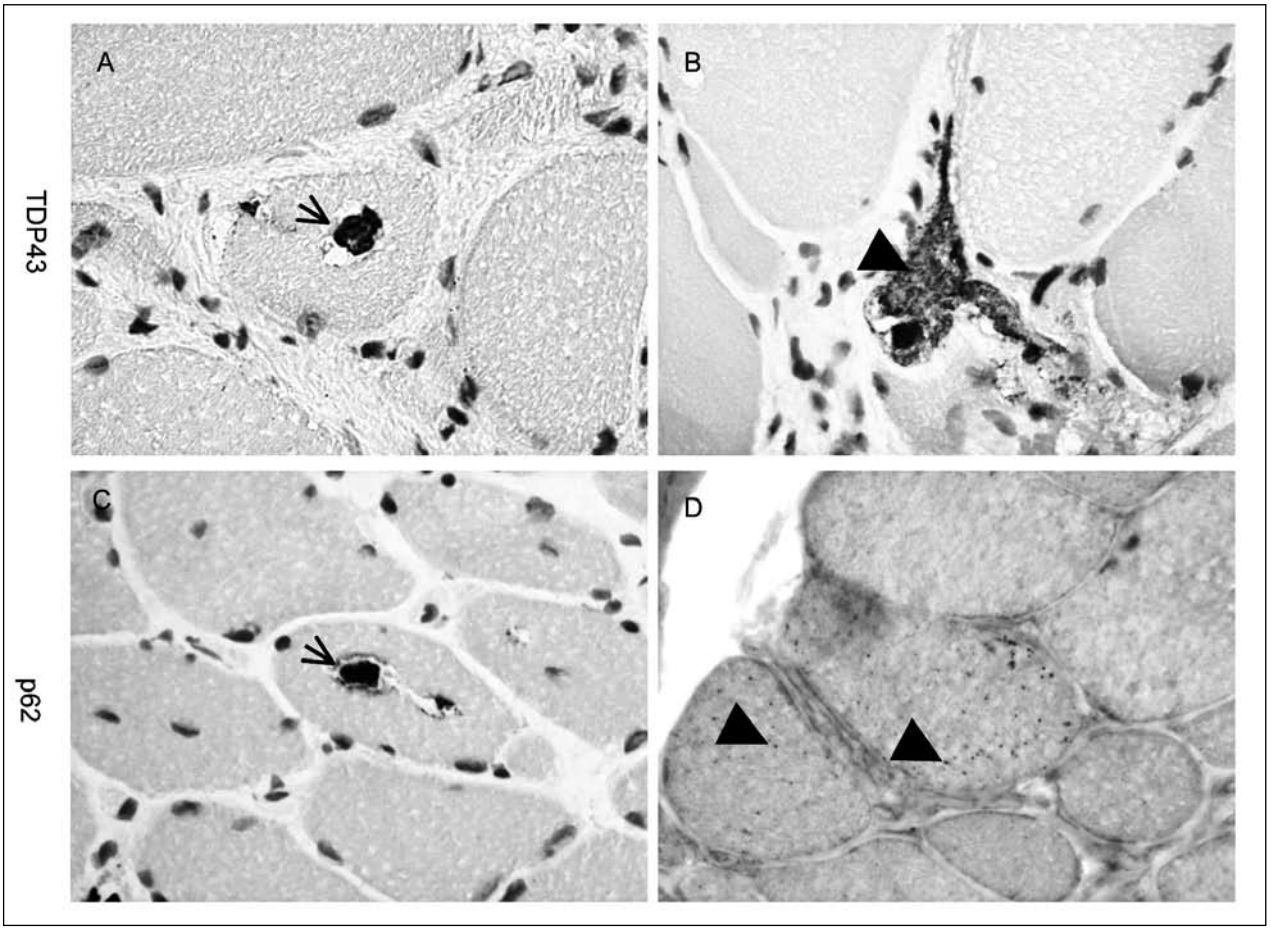

Figure 1. Immunohistochemistry on frozen sections with TDP-43 (A, B) and p62 (C, D) antibodies. TDP-43 and p62 immunoreactivity was present as larger rounded deposits in the vacuole of the vacuolated fibers (A, C, arrow) and/or as small multiple granular aggregates in the non-vacuolated cytoplasm of vacuolated fibers (B, arrowhead) or non-vacuolated fibers (D, arrowhead).

of muscle fibers, vacuolar degeneration and accumulation of multiple proteins (2). Some of the proteins accumulated in vacuolated muscle fibers in s-IBM are also found abnormally accumulated in the brain in Alzheimer disease (AD), suggesting that both diseases could have some common pathogenic mechanisms. These proteins include ubiquitin, amyloid-β (Aβ), amyloid-β precursor protein (AβPP), phosphorylated-tau (p-tau), apolipoprotein E (ApoE) and α_1-antichymotrypsin (8-13). It has also been shown that α-synuclein, cellular prion protein as well as markers of oxidative stress, proteins of the ubiquitine-proteasome system, endoplasmic reticulum chaperones, heat shock proteins, signal transduction components are accumulated in s-IBM muscle fibers (2). The elimination of improperly folded or unfolded proteins is normally ensured in the cell by several mechanisms, including refolding through endoplasmic reticulum chaperones and heat shock proteins, and degradation by the ubiquitine-proteasome system and through autophagosomes formation, all processes which might be impaired in s-IBM. Predisposing genes and an aging cellular milieu may contribute to the dysfunction of these mechanisms, leading to the accumulation of insoluble proteins aggregates.

The extensive description of proteins aggregated in s-IBM has largely contributed to a better understanding of the physiopathology of s-IBM. However, it has not contributed in the same way to the improvement of histological diagnosis. As a matter of fact, difficulties for defining s-IBM by its canonical biopsy features have been stressed by several authors (6, 7). Distinguishing between s-IBM and polymyositis is though of great importance. On one hand, it has been shown from the follow-up of two large cohorts of patients with s-IBM that immunosuppressive treatments seem not to ameliorate the natural course and even so might hasten progression of the disease (14). Therefore, it will be crucial to include patients

with the right diagnosis in future clinical trials aimed at demonstrating the efficacy of treatments. Considering the canonical features mandating for a definite diagnosis in Griggs' criteria, it has been shown that amyloid deposits evidenced by red fluorescence are rarely encountered (15, 16) and electron microscopy to detect 15-21 nm tubulofilaments is not performed routinely. In the past few years, efforts have been made to identify markers of the degenerative process which might be used for diagnosis purposes. These markers are SMI-31, SMI-310, p62 and TDP-43. SMI-31, reacting with a phosphorylated epitope of neurofilament heavy chain and crossreacting with tau, detects tubulofilamentous inclusions. It has been demonstrated to be highly specific and proposed to replace electron microscopy (3). SMI-31 immunoreactive deposits have been identified in 80% of the vacuolated muscle fibers, either within the vacuoles or in the vacuole-free cytoplasm, and occasionally in non-vacuolated fibers (17). This marker was judged to be helpful to differentiate, among patients with biopsies showing inflammation and rimmed vacuoles, treatment responders and non responders (18). Another marker of phosphorylated-tau, SMI-310, was expressed in 60 to 80% of the vacuolated muscle fibers in s-IBM, but not in hereditary inclusion body myopathy (h-IBM), an heterogeneous group of inherited disorders characterized histologically by vacuolated muscle fibers but no inflammation (17). p62, also known as sequestosome 1, is a shuttle protein transporting polyubiquinated proteins for their degradation by both the proteasome and lysosome. This protein is a component of the inclusions in several neurodegenerative disorders, including AD (19). Given the similarities between s-IBM and AD, p62 was studied on s-IBM muscles and shown to be present as linear, squiggly or small rounded aggregates in all specimens, permitting to distinguish between s-IBM and polymyositis (20). Intracellular protein aggregation has been identified in other muscle disorders grouped under the term of "protein aggregate myopathies" (PAM) (21). PAM includes different types of genetic myopathies such as myofibrillar myopathies (MFMs), hereditary inclusion body myopathies (h-IBM), actinopathies and myosinopathies (22). The literature upon PAM has shed light on other proteins that could be involved in the physiopathology of s-IBM. In particular, it has been show that DNA-binding protein 43 (TDP-43) is accumulated in desminopathies and myotilinopathies, subgroups of MFMs caused by mutations in *DES* and *MYOT* genes (21), h-IBM with Paget's disease of the bone and fronto-temporal dementia (23), due to valosin containing protein (VCP) gene mutations and s-IBM (16, 21, 24). TDP-43 is a 414-amino acid nuclear protein, highly conserved and widely expressed in several tissues, implicated in exon skipping, transcription regulation, and other biologic processes through its binding to DNA, RNA, and/or proteins (25). TDP-43 is one component of the ubiquinated inclusions in the brain of patients with frontotemporal dementias and amyotrophic lateral sclerosis (26). According to a recent study, sarcoplasmic immunoreactivity for TDP-43 is highly sensitive and specific of s-IBM among inflammatory myopathies (16).

By our systematic screening, we have demonstrated that TDP-43 and p62 were the more sensitive markers for the diagnosis of s-IBM. These results confirm those of previous reports (16, 20). The immunoreactivity appears either as rounded aggregates within the vacuole or as small multiple granular aggregates in the vacuolated-free cytoplasm of vacuolated fibers or in non-vacuolated fibers. The originality of our approach was to compare the results in two groups with either presumed *d* s-IBM or *p* s-IBM. We used the term "presumed" because detection of amyloid deposits and/or detection of PHF was not performed routinely, and thus, our criteria for *d* s-IBM did not fulfilled strictly Grigg's criteria. Nevertheless, detection of p62 aggregates in all presumed *d* s-IBM cases confirm *a posteriori* that our diagnosis was valid because p62 has been shown by immunoelectronmicroscopy to co-localize with bundles of PHF (20). Our study shows that the use of p62 and TDP-43 permits to re-classify 37% and 31%, respectively, of our *p* s-IBM diagnosed with conventional stain as presumed *d* s-IBM. This percentage was increased to 42% by adding the cases re-classified with each marker. This information is very useful for the clinician to guide the therapeutic options. In our hands, the percentage of immunoreactive fibers in presumed *d* s-IBM and *p* s-IBM was similar for both markers. Concerning the specificity, we have shown that TDP-43 immunoreactivity was observed neither in pathological nor in normal control muscle biopsies. TDP-43 positive inclusions were found in 1 of 12 steroid responsive polymyositis in another study (23). p62 aggregates were identified as small diffuse speckles in the cytoplasm of numerous fibers in one case of polymyositis (20). The pattern of immunoreactivity was quite different than that observed in s-IBM. Similar observation has been mentioned in rare fibers in polymyositis, dermatomyositis and non-specific myopathies (20). We suggest that both markers could be used in the histological diagnosis workup of s-IBM. The contribution of these markers to the diagnosis of s-IBM has to be further validated in prospective series.

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