

Clinical, morphological and genetic studies in a cohort of 21 patients with myofibrillar myopathy

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The term myofibrillar myopathies (MFM) refers to uncommon neuromuscular disorders that pathologically are characterized by myofibrillar degeneration and ectopic expression of several proteins. MFM are partly caused by mutations in genes that encode mainly Z-disk-related proteins (desmin, α B-crystallin, myotilin, ZASP, filamin C and BAG3). We reviewed clinical, light and electron microscopy, immunohistochemistry, immunoblotting and genetic findings of 21 patients with MFM (15 unrelated patients and three pairs of brothers) investigated at our neuromuscular center. MFM patients begin to show symptoms at any age, from juvenile to late adult life and present a different distribution of muscle weakness. Cardiac involvement and peripheral neuropathy are common. Typical histological features include focal areas with reduction/loss of ATPase and oxidative enzyme activity, and amorphous material (eosinophilic on hematoxylin and eosin and dark blue on Engel-Gomori trichrome) in these abnormal fiber areas. Electron microscopy shows disintegration of myofibrils starting from the Z-disk and accumulation of granular and filamentous material among the myofilaments. Immunohistochemical studies demonstrate focal accumulation of desmin, α B-crystallin and myotilin in abnormal muscle fibers while immunoblot analysis does not highlight differences in the expression of these proteins also including ZASP protein. Therefore, unlike immunoblot, immunohistochemistry together with light and electron microscopy is a useful diagnostic tool in MFM. Finally three of our 21 patients have missense mutations in the desmin gene, two brothers carry missense mutations in the gene encoding myotilin, one has a missense mutation in α B-crystallin, and none harbour pathogenic variations in the genes encoding ZASP and BAG3.

Key words: Myofibrillar myopathies, desmin, α B-crystallin, myotilin, Z-band alternatively spliced PDZ motif containing protein (ZASP), filamin C, Z-disk

Introduction

Myofibrillar myopathies (MFM) are uncommon inherited or sporadic progressive neuromuscular disorders with clinical and genetic heterogeneity (1, 2). MFM are morphologically defined by foci of myofibril dissolution, accumulation of myofibrillar degradation products, and ectopic expression of multiple proteins including desmin, α B-crystallin, dystrophin, myotilin, sarcoglycans, neural cell adhesion molecule (NCAM), plectin, gelsolin, ubiquitin, filamin C, and congophilic amyloid material (3-5).

To date, mutations in six genes are known to cause MFM, but these account for less than half of patients with a diagnosis of MFM (1). These genes encode mainly sarcomeric Z-disk or Z-disk-related proteins and the mutated proteins are usually found inside the aggregates: desmin (6), α B-crystallin (7), myotilin (8), Z-band alternatively spliced PDZ motif containing protein (ZASP) (9) and filamin C (10). Additionally, mutations in BAG3 have recently been shown to cause MFM (11). Despite the identification of several mutations in different genes, the typical histological features are observed in all patients (12). To date, the mechanisms leading to protein aggregation are not fully understood and recent studies proposed that the fiber abnormalities in MFM probably are a common step of a stress-induced pathway, triggered by different stimuli (13, 14).

We here describe our clinical, light and electron microscopy, immunohistochemistry, immunoblotting and genetic analysis findings in 21 MFM patients investigated at our neuromuscular center.

Patients and methods

Patients

Twenty-one patients were diagnosed as affected with MFM at our neuromuscular center. The cohort included 15 unrelated patients and three pairs of brothers. Patients were studied at clinical, morphological, biochemical and genetic level; clinical and genetic studies were done in all 21 cases and muscle biopsy was performed in 20 patients.

Histology and histochemistry

Muscle samples were snap frozen in liquid nitrogen-cooled isopentane. Serial 8- μ m-thick cryosections were stained with haematoxylin and eosin (H&E), Engel-Gomori trichrome, adenosine triphosphatase (ATPase, pre-incubation at pH 4.3, 4.6 and 10.4), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), reduced nicotinamide adenine dinucleotide (NADH), periodic acid-Schiff (PAS) with diastase digestion, Sudan black and acid phosphatase.

Electron microscopy

A small fragment of muscle tissue was fixed in 4% glutaraldehyde in phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated and embedded in Spurr resin. Semithin sections were stained with toluidine blue and PAS. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 electron microscope.

Immunohistochemical studies

Immunohistochemistry was performed on serial 6.5- μ m-thick sections with antibodies to desmin, α B-crystallin and myotilin; the reactions were revealed by immunofluorescence methods, as previously described (15). Controls were muscle biopsies from subjects who were ultimately deemed to be free from muscle diseases. To control staining specificity the primary antibody was omitted or replaced with nonimmune sera at the same concentration.

SDS-PAGE electrophoresis and immunoblot analysis

The expression of desmin, α B-crystallin, myotilin and ZASP was evaluated by one-dimensional immunoblotting as described (15). Briefly, twenty 20- μ m-thick frozen muscle sections were homogenized with a lysis buffer (50 mM

Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% deoxycholic acid, pH 7.5 and including protease inhibitors), sonicated and centrifuged at 1500 g for 10 min. Protein concentration was determined in the supernatants with the Bradford method. Aliquots corresponding to 20 μ g of proteins were loaded on a 10% T polyacrylamide gel and separated by electrophoresis. Samples were transferred to a nitrocellulose membrane that was blocked with non-fat dried milk for 60 min at room temperature and incubated overnight at 4°C with the specific antibodies. After intervening washes, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Bands were visualized with the ECL Advance Western Blotting Detection Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Protein loading was confirmed by Coomassie staining of the gel. Bands were quantified densitometrically using the Quantity One software (Bio-Rad).

Genetic studies

DNA was extracted by standard methods from the whole blood obtained after informed consent for diagnostic procedures. In all MFM patients was performed at genomic level the screening for mutations in the following genes: Desmin (DES, NCBI Reference Sequence: NG_008043.1), Myotilin (MYOT, NCBI Reference Sequence: NG_008894.1), Crystallin, alpha B (CRYAB, NCBI Reference Sequence: NG_009824.1) LIM domain binding 3 (LDB3, NCBI Reference Sequence: NG_008876.1) and BCL2-associated athanogene 3 (BAG3, NCBI Reference Sequence: NG_016125.1).

We PCR amplified all the coding exons, the splice site junctions and 100bp of flanking introns (primers sequences available upon request); PCR reactions were carried out in a volume of 25 μ l containing 25 ng of DNA template and 2.5U Taq polymerase (Invitrogen), 1X Buffer, 0.75 mM MgCl₂, 200 μ M dNTPs, 0.5-1 μ M each primer.

The amplification conditions were as follows: 94°C initial denaturation (5 min), 94°C denaturation (45s), 60°C (45s) annealing, 72°C (60s) extension for 30 cycles, 72°C (7 min) final extension.

The amplified PCR products were analyzed by direct sequencing on an automatic sequencer (ABIPRISM 3130, Applied Biosystems, Foster City, CA). All the sequence variations were annotated taking the first base of the Met codon counted as position 1 in the Reference Sequence.

Results

Clinical characteristics of patients

A total of 21 MFM patients from 18 families were studied. The male to female ratio was 1.6; thirteen pa-

tients were men and eight were women. The age of disease onset ranged from 16 to 78 years (mean, 42.8 years; median 55 years) and the age at diagnosis varied from 30 to 79 years (mean, 52.1 years, median 53 years). Seven patients reported a family history suggestive of neuromuscular disorders and among these three pairs of brothers were present.

The symptoms reported at the time of muscle biopsy consisted of muscle fatigue, 12; slowly progressive weakness, nine; muscle wasting, seven; myalgia and/or cramps, six; myotonia, one; dysphagia and/or dysphonia, four; dyspnoea, four; palpitations, four; paresthesias and/or hypoesthesias, seven.

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Clinical examination revealed muscle weakness in 17 patients; the remaining four patients complained of myalgias associated with elevated serum CK (three patients) or had similarly affected family members (one patient). The distribution of weakness at the time of diagnosis was different in the 17 patients. In four, the weakness involved only axial muscles; two patients had weakness of the paravertebral muscles both at thoracic and at cervical level; the other two presented weakness of the paravertebral muscles at thoracic level. Thirteen patients had weakness of limb muscles; in eight, the weakness involved both proximal and distal limb muscles; in four, the weakness was only proximal, and in two it was only distal. At follow-up evaluation, in two patients the axial muscle weakness spread to limb muscles; in five weakness localized to the proximal or distal limb at the time of diagnosis, later extends distally or proximally.

Among 10 patients who underwent to heart assessment, seven had cardiac involvement. Two of these patients had signs of congestive heart failure and arrhythmia; two had signs of congestive heart failure without arrhythmia; and three had arrhythmia without congestive failure. Arrhythmias included paroxysmal supraventricular tachycardia in three patients, left bundle branch block in two affected brothers with desmin mutation, and sinus block in one patient. Two patients with arrhythmias underwent pacemaker implantation.

Serum CK was determined in 12 patients and the mean level was found to be 4-fold above the upper normal limit (range, from normal to 9-fold).

Nerve conduction studies and electromyography (EMG) was performed in 18 patients. Abnormal spontaneous activity consisting of fibrillation potentials, positive sharp waves and complex repetitive discharges was recorded in three patients; one of these (the patient with the clinical symptom of myotonia) had also myotonic discharges. Ten patients had low-amplitude, short-duration polyphasic motor unit potentials (MUPs); two high-amplitude, long-duration MUPs and four normal MUPs.

Histology and histochemistry

Histological analysis documented myopathic features in all specimens. Muscle biopsies from all MFM patients showed areas with a loss or reduction of ATPase

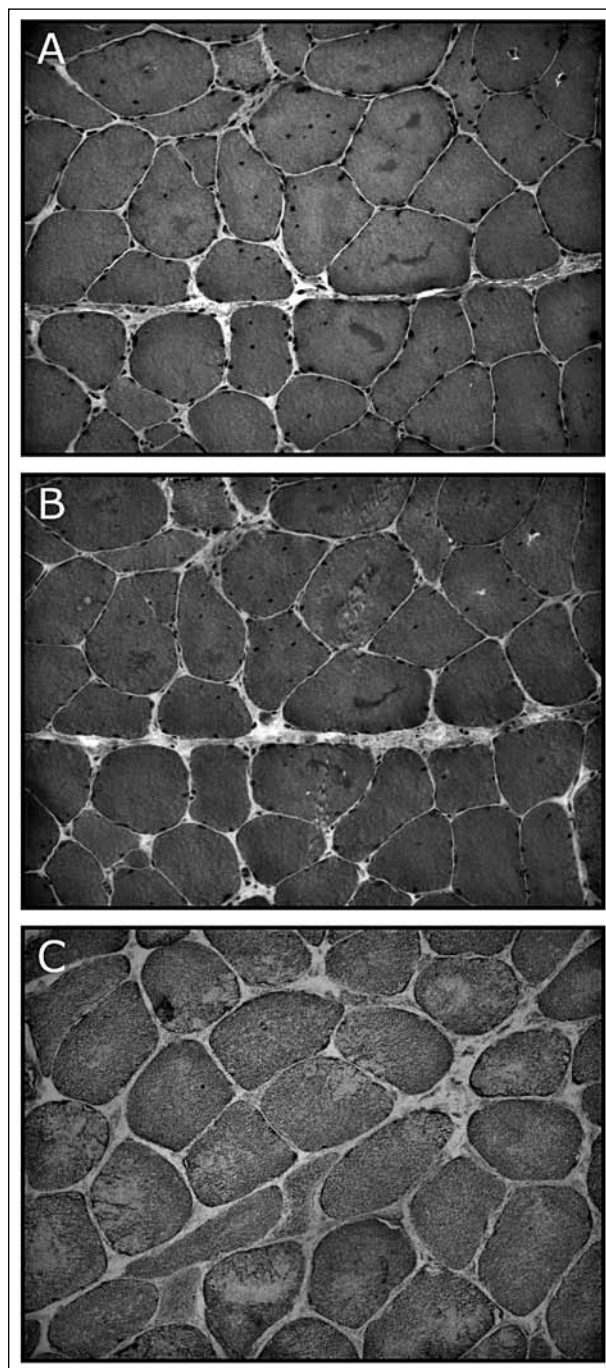


Figure 1. Histological findings in myofibrillar myopathies. Muscle fibers with amorphous material that stains eosinophilic on hematoxylin and eosin (A) and dark blue on Engel-Gomori trichrome (B). Several muscle fibers with loss of NADH activity (C).

and oxidative enzyme activity (COX, SDH and NADH) (Fig. 1). These abnormal areas had variability in number, shape and size, were randomly distributed inside the muscle fibers. Many abnormal fiber regions harboured amorphous material that was eosinophilic on hematoxylin and eosin staining and dark blue on Engel-Gomori trichrome (Fig. 1). Slight fiber size variation was observed in 14 patients. Atrophic angulated fibers, singly or in small groups, were of either histochemical type and occurred in 13 specimens. Type 1 fiber predominance was found in five cases and type 2 fiber predominance only in one patient. Sparse small vacuoles, rimmed or not, appeared in half of the specimens; cytoplasmic bodies were infrequent but in one patient they were the only histological abnormality. Necrotic fibers were rare and isolated on a given section and were present in five cases, in five muscle biopsies there was also fiber degeneration. Regenerating fibers were uncommon (three cases). In one biopsy specimen there was an endomysial inflammatory infiltrate. Increase of endomysial connective tissue with occasional adipocyte infiltration was observed in eight patients. Internal nuclei and/or fibers subdividing by splitting were present in four cases. The muscle fiber lipid and glycogen content was normal.

Electron microscopy

Ultrastructural examination showed single or multiple focal areas of different size with myofibrillar disruption, streaming of Z-disk, and abundant granulo-filamentous material which was either diffusely interspersed among the myofibrils or located under the sarcolemma (Fig. 2). On longitudinal sections, the granulo-filamentous material seemed to derive from Z-disk; occasionally it was aggregated to form spherical structures. Vacuoles

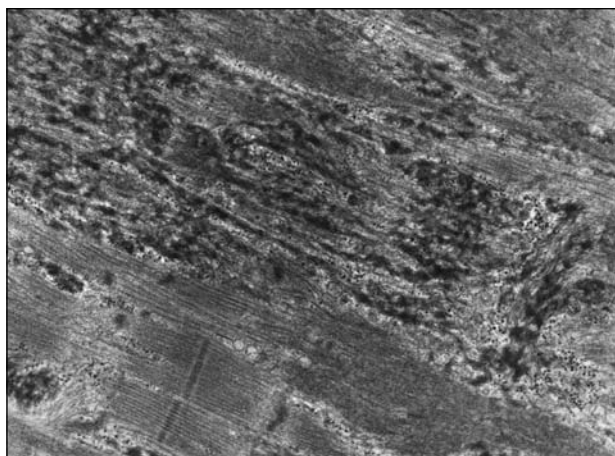


Figure 2. Ultrastructural features of myofibrillar myopathies. Granulo-filamentous material diffusely interspersed among the myofibrils.

containing cytoplasmic degradation products were common and typical cytoplasmic bodies were rare. There was no evidence of ultrastructural abnormalities involving other cellular structures, in particular nuclei, T-tubules and sarcoplasmic reticulum.

Immunohistochemistry

In all muscle biopsies from patients with MFM, abnormal fibers with focal areas of increased reactivity for desmin, α B-crystallin and myotilin were observed (Fig. 3). These areas were single or multiple, variable in shape and size and unevenly distributed within the fibers. The percentage of abnormal fibers was variable between the different cases examined and ranged between 2 and 15% of the total muscle fibers. The α B-crystallin deposits resembled the desmin and myotilin deposits in distribution and staining intensity but they appeared to be more frequent than the other two proteins.

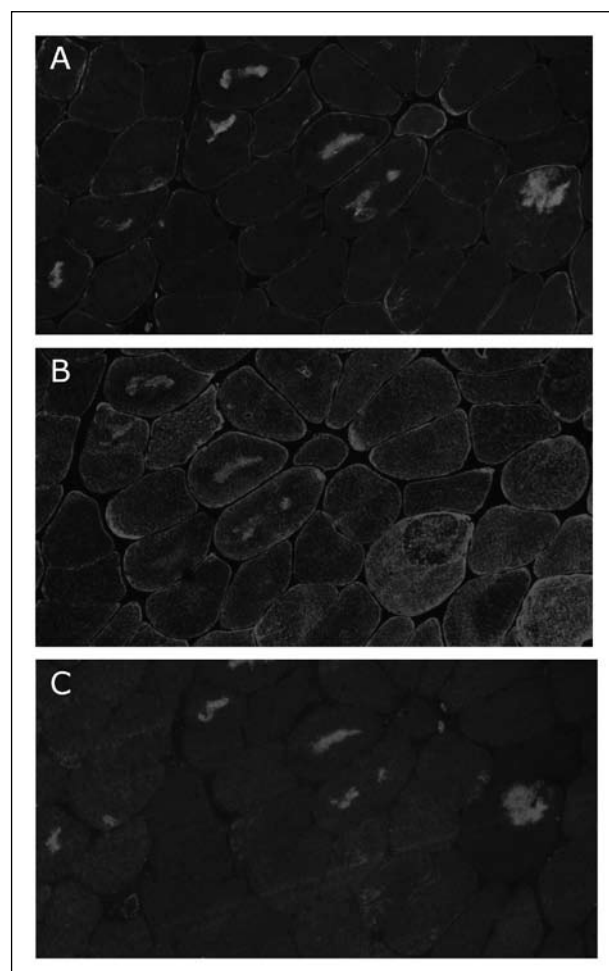


Figure 3. Immunohistochemistry for α B-crystallin, desmin and myotilin in myofibrillar myopathies. Focal accumulation of α B-crystallin (A), desmin (B) and myotilin (C) in several muscle fibers.

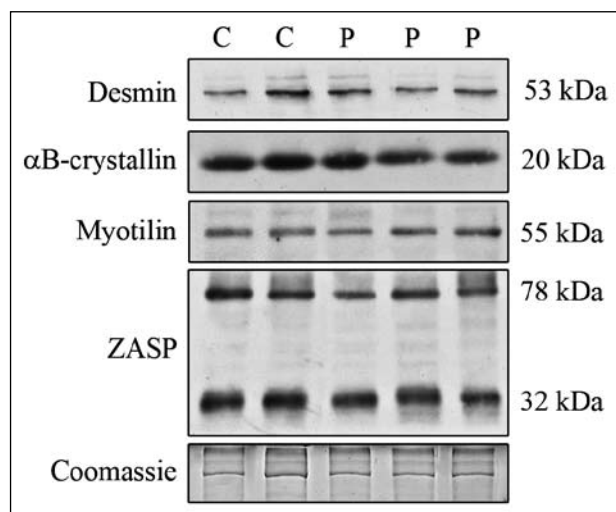


Figure 4. Immunoblot for desmin, α B-crystallin, myotilin and ZASP. The intensity of all bands was similar in both patients' (P) and controls' (C) muscles. Coomassie blue staining was used to assess protein loading.

SDS-PAGE electrophoresis and immunoblot analysis

In normal and MFM muscle biopsies desmin migrated as a 53 kDa band, α B-crystallin as a 20 kDa band and myotilin as a 55 kDa band. ZASP protein instead showed two bands at the molecular weight of 78 and 32 kDa. The intensity of all bands was similar in muscle of patients and control subjects (Fig. 4).

Genetic Studies

In the Desmin gene we identified in two affected brothers the c. 20C > T substitution in heterozygosis (S7F) previously unreported and in another patient the c. 1360C > T substitution in heterozygosis (R454W) reported as pathogenetic (16). The Crystallin, alpha B gene analysis revealed in one patient in heterozygosis the c.460G > A mutation (G154S) reported as pathogenetic (17). In two affected brothers the analysis of myotilin gene showed the known pathogenetic mutation c. 116C > T (S39F) in heterozygosis (18). None of our MFM patients harboured pathogenetic variations in the LIM domain binding 3 gene and in the BCL2-associated athanogene 3. In all the genes analyzed we identified several known polymorphism both in heterozygosis and in homozygosis.

Discussion

We present here a retrospective analysis of data derived from 21 patients with MFM investigated at our neuromuscular center. MFM patients begin to show symptoms at any age, from juvenile to late adult life and diagnosis is delayed compared to the age of the first appearance of the

symptoms. In our cohort onset was not observed until after reaching 16 years of age. From clinical point of view, we should emphasize that symptoms reported at the time of diagnosis are extremely variable and muscle weakness is not documented in all our patients at the time of being subjected to muscle biopsy. Cardiac involvement is common occurring in 39% of our patients. There is an increased incidence of electrocardiographic abnormalities, including atrial arrhythmias, atrioventricular conduction defects and bundle branch block. Congestive heart failure is not uncommon; in our series it was documented in 4 of 21 patients. In our study we also found a frequent involvement of peripheral nervous system. Clinical signs and/or EMG findings of peripheral neuropathy are present in approximately 44% of our cohort and even in one patient sensory deficits are presenting symptoms.

A broad spectrum of light microscopic changes ranging from mild to severe abnormalities was documented in all muscle biopsy specimens of our patients. The typical pathological findings include: (1) focal areas with either reduction/loss of NADH, SDH and COX staining or decreased ATPase activity present in almost all patients; (2) amorphous material that stains eosinophilic on hematoxylin and eosin and dark blue on Engel-Gomori trichrome in most of these abnormal fiber areas; (3) rimmed and non-rimmed vacuoles observed in 45% of cases and cytoplasmic bodies in only 10%. In addition to myopathic features, the presence of small angulated fibers of either histochemical type which may occur singly or in groups suggest a neurogenic process; these findings were present in more than 60% of patients. Although none of these histological abnormalities is specific for MFM, the combination of these changes outline the diagnosis (2). Electron microscopic studies documented disintegration of myofibrils starting from the Z-disk and accumulation of granular and filamentous material widely distributed among the myofilaments. These abnormalities observed at ultrastructural level suggested the temporal sequence of pathological events occurring in MFM (12). Initially there is a breakdown of myofibrils beginning at the Z-disk owing to loss of the normal architecture of the myofibrils and disruption of intermediate filaments; then deposits of filamentous material accumulate in different shape and size displacing membranous organelles; and ultimately membranous organelles are sequestered in autophagic vacuoles.

Our immunohistochemical data demonstrated the focal accumulation of desmin, α B-crystallin and myotilin in muscle fibers of all patients and thus confirm that immunohistochemistry is a sensitive tool to detect protein aggregation in MFM muscle specimens (2). These deposits correspond to the abnormal fiber regions without, or with reduced, ATPase and/or oxidative enzyme activity

and are highly variable in terms of number, shape, size and intracellular localization. While in some specimens protein aggregates are multiple, widespread within muscle fibers and affect up to 15% of all fibers, in others these inclusions are singly, tiny and only in a small percentage of fibers. In most cases aggregates are immunoreactive for all three proteins but α B-crystallin deposits are more numerous than the other two.

In contrast to light and electron microscopy, immunoblot analysis is not useful and informative for the diagnosis of MFM because in muscle of MFM patients it does not highlight differences in the expression of mostly commonly proteins observed in the intracellular deposits. These data seem to suggest, therefore, that the total content of proteins in MFM muscle might be unchanged when compared to normal controls and are in agreement with the previous demonstration that in patients with MFM there is no increased gene transcription of different proteins that are believed involved in the pathogenesis of this disease including desmin and α B-crystallin (19).

Finally we identified genetic mutations in six out of 21 patients with MFM: three from two families had different mutations in the desmin gene while in three other cases, which also belonged to two different families, mutations in the genes encoding myotilin (in two affected brothers) and alpha crystallin B chain were found. Instead in our cohort we did not find any pathogenic variations in the genes encoding LIM domain-binding protein 3 and BAG3. One of the four reported mutations was never described in the literature and in particular the missense mutation S7F in the desmin gene identified in two affected brothers; the other three missense mutations including one in the gene encoding desmin (R454W), one in the myotilin gene (S39F) and the other in the gene of α B-crystallin (G154S) have already been documented and considered as pathogenetic (16-18). Therefore, the genetic basis of MFM was established in only 28% of our cases and these data are consistent with those reported in other studies (20).

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