

NEMALINE MYOPATHY

Clinical, histochemical and immunohistochemical features

Nazah Cherif Mohamad Youssef, Rosana Herminia Scola, Paulo José Lorenzoni, Lineu César Werneck

Abstract – Nemaline myopathy (NM) is a congenital disease that leads to hypotonia and feeding difficulties in neonates. Some cases have a more benign course, with skeletal abnormalities later in life. We analyzed a series of eight patients with NM obtained from a retrospective analysis of 4300 muscle biopsies. Patients were classified as having the typical form in five cases, intermediate form in two cases and severe form in one case. Histochemical analysis showed mixed rods distribution in all cases and predominance of type I fibers in five cases. Immunohistochemical analysis showed abnormal nebulin expression in all patients (four heterogeneous and four absent), homogeneous desmin expression in four cases, strongly positive in three and absent in one, fast myosin expression in a mosaic pattern in six cases and absent in two cases. There was no specific relation between these protein expression patterns and the clinical forms of NM.

KEY WORDS: nemaline myopathy, nebulin, desmin, myosin, immunohistochemistry.

Miopatia nemalínica: achados clínicos, histoquímicos e imuno-histoquímicos

Resumo – Miopatia nemalínica (NM) é uma doença congênita que leva a hipotonia e dificuldade de sugar em neonatos. Alguns casos possuem uma evolução benigna, com deformidades ósseas tardias. Nós analisamos uma série de oito pacientes com NM obtidos da análise retrospectiva de 4300 biópsias musculares. Os pacientes foram classificados como forma típica em cinco casos, forma intermediária em dois casos e forma severa em um caso. Análise histoquímica mostrou distribuição mista dos rods em todos os casos e predominância de fibras tipo I em cinco casos. Análise imuno-histoquímica mostrou expressão anormal da nebulina em todos os pacientes (quatro heterogênea e quatro ausente), expressão homogênea da desmina em quatro casos, fortemente positiva em três e ausente em um, expressão da miosina (rápida) com padrão em mosaico em seis casos e ausente em dois casos. Não há relação específica entre a expressão destas proteínas e as formas clínicas da NM.

PALAVRAS-CHAVE: miopatia nemalínica, nebulina, desmina, miosina, imuno-histoquímica.

Nemaline myopathy (NM) is a congenital myopathy first described in 1963 by Shy and Conen, characterized by hypotonia and general muscle weakness predominantly in facial muscles, cervical and trunk flexor muscles, foot dorsiflexor muscles and finger extensor muscles^{1,2}. Patients may also exhibit dysphagia, respiratory insufficiency, foot deformities, arch palate, scoliosis, chest deformities and superior and inferior limbs contractures¹⁻⁷. Currently, NM is classified into six different forms: severe congenital, typical, intermediate congenital, juvenile, adult and other forms associated with cardiomyopathy, ophthalmople-

gia and the presence of intranuclear nemaline bodies on muscle biopsy⁸. Adult forms are known as sporadic late-onset nemaline myopathies (SLONM) and are associated with HIV and monoclonal gammopathy^{9,10}.

An NM diagnosis is made with a muscle biopsy in which modified Gomori-trichrome (MGT) staining shows the presence of nemaline bodies in muscle fibers in the subsarcolemmal or intermyofibrillar region^{1,2,5,11,12}. The histochemistry also reveals features in NM such as a predominance of type I fibers and an increase in acid phosphatase activity^{5,12-14}. An immunohistochemical study of

Neuromuscular Disorders Service, Neurology Division, Internal Medicine Department, Hospital de Clínicas da Universidade Federal do Paraná (UFPR), Curitiba PR, Brasil.

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Dra. Rosana Herminia Scola – Serviço de Doenças Neuromusculares / Hospital de Clínicas da UFPR – Rua General Carneiro 181 / 3º andar - 80060-900 Curitiba PR - Brasil. E-mail: scola@hc.ufpr.br

muscle proteins shows the nature of the structural failures in NM. Several studies have shown negative and positive expression of nebulin, myosin, desmin, titin and dystrophin in muscle fiber and rods^{5,15-18}. Nemaline myopathy remains genetically heterogeneous. To date, defects in six distinct genes have been identified in cases of NM. These include mutations in α -tropomyosin (TPM3), beta-tropomyosin (TPM2), nebulin (NEB), α -actin (ACTA1), cofilin2 (CFL2) and troponin T (TNNT1)^{4,17-28}.

This study describes the clinical manifestations of nemaline myopathy using histochemical analysis along with expression analysis of nebulin, desmin and myosin proteins with immunohistochemistry.

METHOD

A retrospective analysis of 4300 muscle biopsies performed between January 1978 and July 2007 identified 15 patients with a diagnosis of NM, but seven cases were excluded because there was not enough material from the muscle biopsy for immunohistochemical analysis. Relevant data, including the clinical evaluation, serum enzymes levels, needle electromyography (EMG), and histological-immunohistochemical aspects of the each muscle biopsy, were collected. Consent to analyze the muscle biopsies was obtained in the out-patient clinic or during hospital admission for diagnostic investigation.

Clinical evaluation

Patients were classified as typical form, intermediate form or severe form, according to criteria established by the International Nemalin Myopathy Consortium⁸. Particular attention was paid to obstetrical history, pregnancy complications, muscle weakness and tone, breastfeeding difficulties, respiratory failures, dysphagia, and facial and skeletal deformities (dysmorphisms). Other relevant data were also collected including age, gender, course of disease and family history.

Laboratory analysis

The levels of creatine kinase (CK), aldolase (AL) and lactate dehydrogenase (LDH) were recorded as a proportion reflecting their increase above normal limits.

Electromyographic findings

The electromyography (EMG) pattern was classified as normal, myopathic, denervation or mixed (myopathic with denervation findings) according to standard procedures²⁹.

Muscle biopsy

Muscle biopsies were frozen in liquid nitrogen at -170°C and cryostat sections were stained histologically, according to standard procedures⁷¹. The predominance of type I fibers with an ATPase reaction (pH 9.4, 4.6 and 4.3), moth-eaten fibers with NADH-tetrazolium reductase, focal increases in acid phosphatase, and the frequency and distribution (subsarcolemmal, intermyofibrillar

or mixed) of fibers with rods on MGT were determined by counting approximately 300 to 500 muscle fibers of each specimen.

Immunohistochemical analysis

The cryostat sections (4 μm) of muscle biopsies were used for immunohistochemistry with specific primary antibodies and secondary antibody (anti-IgG) conjugated to fluorescein for nebulin, desmin and myosin proteins, according to standard procedures. The primary antibodies used were an anti-nebulin monoclonal antibody (Sigma, dilution 1:50), an anti-desmin monoclonal antibody (Sigma, dilution 1:20) and a fast anti-myosin monoclonal antibody (Sigma, dilution 1:10). The secondary antibody conjugated to fluorescein was used at a dilution 30:500. Afterwards, an immunofluorescence analysis was performed using an epifluorescence microscope. The immunofluorescent classification was modified based on the descriptions of Gurgel-Giannetti et al.¹⁵ and Imoto et al.¹⁶: (1) nebulin: absent expression, heterogeneous expression (diffused imperfection), or homogeneous expression; (2) desmin: absent expression, homogeneous expression, or strongly positive expression; and (3) fast myosin: absent expression or mosaic-type expression.

RESULTS

There were eight patients (six female and two male) aged 2 months to 6 years, with a mean age of 22 months. The patients were classified as having the typical form in five cases, the intermediate form in two cases and severe form in one case. Muscle hypotonia since birth was present in all cases. The other clinical findings reported were skeletal or facial dysmorphisms, breastfeeding difficulties and respiratory failure (Table). Dysphagia, cardiomyopathy and ophthalmoplegia were not observed. No obstetric abnormalities were observed in six cases, but gestational complications such as a decreased fetal movements, oligohydramnios and premature birth were observed in two cases (intermediate form and severe form). No family history was observed (Table).

Only one case (case 4) had serum CK and LDH levels increased above the normal limits (Table).

The EMG pattern was observed in seven patients and was myopathic in two cases, neurogenic in three cases and normal in the remainder. The EMG revealed abnormalities in four cases of the typical form, two of the intermediate form and in the one of the severe congenital case (Table).

A muscle histological analysis was performed in all cases and the most common abnormalities found in muscle biopsies were rods in all cases, type I fiber predominance in five cases, moth-eaten fibers in four cases and a focal increase in fibers of acid phosphatase activity in four cases (Fig 1 / Table). A frequency of rods above 50% with MGT staining was found in two patients, while a frequency below 50% was found in six patients (Table). The

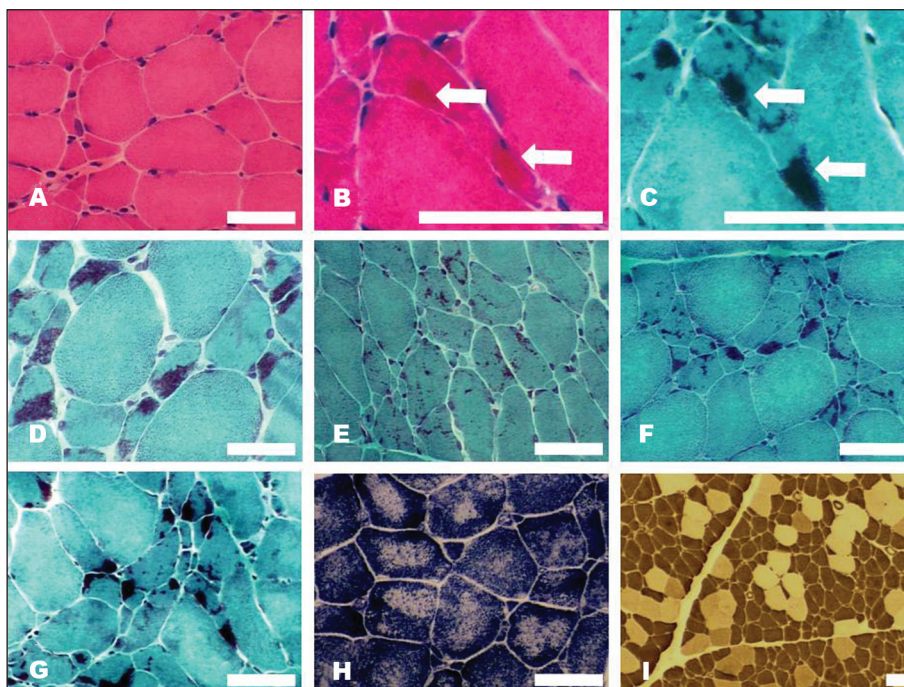


Fig 1. Hematoxilin-Eosin (HE) stain showing variation in fiber sizes with areas of small groups of atrophic muscle fibers [A]. Some of the subsarcolemmal rods are seen with HE (arrow), but become more evident when stained with modified Gomori trichrome (MGT) (arrow) [B and C] The predominant subsarcolemmal (D), intermyofibrillar [E] or mixed rods [F and G], with MGT staining. A histochemical reaction for NADH shows the presence of moth-eaten fibers [H]. The predominance of type I fibers was seen with an ATPase reaction (pH 4.3) [I]. (A, D, E, F, H, I: case 3; B, C, G: case 8). Bar = 50µm.

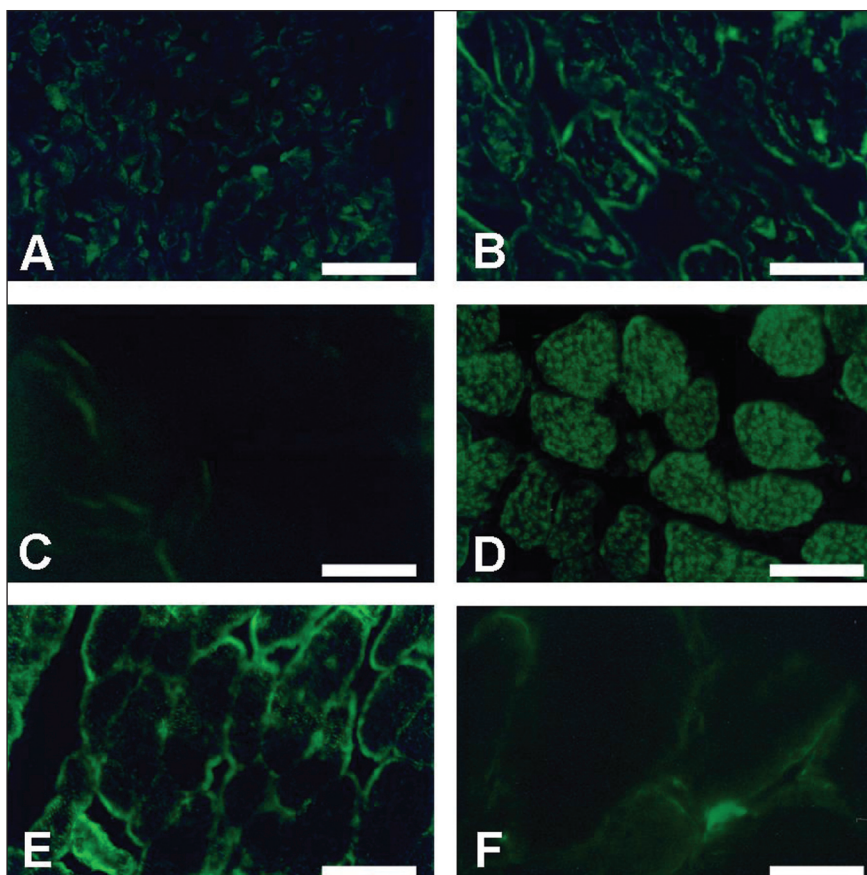


Fig 2. Immunofluorescence changes in muscle fibers with nemaline myopathy: [A/B] focal faults of nebulin, [C] absence of nebulin, [D] normal myosin (mosaic pattern), [E] strongly positive desmin, and [F] normal desmin. Bar = 50 µm.

Table. Relationship between the clinical form and characteristics of NM patients.

	NM classification			Total
	Typical	Intermediate	Severe	
Number of patients	5	2	1	8
Clinical features				
Muscle hypotonia	5	2	1	8
Breastfeeding difficulties	0	2	1	3
Respiratory failure	0	0	1	1
Dysmorphisms	1	1	1	3
Dysphagia	0	0	0	0
Cardiomyopathy	0	0	0	0
Ophtalmoplegia	0	0	0	0
Obstetrical history / Gestational complications				
Normal pregnancy	5	1	0	6
Premature births	0	1	1	2
Oligohydramnios	0	1	0	1
Decreased fetal movements	0	1	1	2
Laboratory findings				
CK increase	0	1	0	1
LDH increase	0	1	0	1
Electromyography pattern				
Myopathy	1	0	1	2
Neurogenic	1	2	0	3
Normal	2	0	0	2
Histological abnormalities	4	1	0	5
Predominance of type I fibers	3	1	0	4
Moth-eaten fibers	3	1	0	4
Focal increase in acid phosphatase in fibers	1	1	0	2
Rods >50%	4	1	1	6
Rods <50%				
Immunohistochemical expression				
Nebulin				
Absent	3	1	0	4
Homogeneous	0	0	0	0
Heterogeneous	2	1	1	4
Desmin				
Absent	1	0	0	1
Homogeneous	3	0	1	4
Strongly positive	1	2	0	3
Myosin				
Absent	2	0	0	2
Mosaic pattern	3	2	1	6

NM: nemaline myopathy, CK: creatine kinase, LDH: lactate dehydrogenase.

distribution of rods was mixed (subsarcolemmal and intermyofibrillar) in all cases (Fig 1).

Immunohistochemical analysis showed abnormal results for nebulin, myosin and desmin muscle proteins in all cases (Table). Nebulin showed heterogeneous expression in four cases and was absent in the other four cases (Fig 2 / Table). We never observed homogeneous expression of nebulin. Desmin expression was homogeneous in four cases, strongly positive in three cases, and absent in

one case (Fig 2 / Table). Fast myosin expression showed a mosaic pattern in six cases and was absent in two cases (Fig 2 / Table). The relationship between the clinical form of NM and nebulin, desmin and fast myosin expression on muscle biopsy is shown in Table.

DISCUSSION

NM is a rare form of congenital myopathy. The small number of cases found in our study is illustrative of the

low incidence of NM of 1 case per 500000 live births²⁵. We identified a higher number of female patients than male patients (six female and two male), although the literature does not suggest any gender predominance.

In the series described by Gommans et al. and Wallgren-Pettersson, there was a higher incidence of the typical form of NM^{3,6}. Although this is a rare disease, the typical form has the highest incidence, followed by the intermediate congenital form and the severe congenital form.

In this study, all of the patients showed mild to severe hypotonia from birth onward, confirming reports of other series of patients with NM^{1-3, 6,7,13}.

With regard to muscle enzymes, CK was normal or slightly elevated (up to five times the normal value), and there was no muscle necrosis or muscle regeneration pattern detected in biopsies of NM patients. This has also been observed in most other cases^{5,6}.

The EMG examination typically shows diverse features in NM patients monitored throughout the course of the disease^{6,29}. Before the age of 3 years, the EMG usually shows mild abnormalities and a myopathic pattern (the motor unit potential (MUP) has a short duration, low amplitude, increased recruitment and short polyphasic potentials) can be found in a few patients. Between the ages of 3 and 10 years, there is an increase in the incidence of abnormalities, although there are many normal MUPs, myopathic MUPs are predominant. After the age of 11 years, there are few normal MUPs and a neurogenic pattern (MUPs with long duration, high amplitude, decreased recruitment and long polyphasic potentials) can occur. In adults with NM, these features are primarily seen in distal muscles such as the tibialis anterior. Spontaneous muscle activity such as fibrillation and positive waves are rare in NM. These abnormalities suggest a progressive form of primary myopathy probably due to motor unit remodeling leading to secondary neuropathy changes. In our cases, the patients with normal EMG were under 2 years old, consistent with the literature. The patients with myopathic and neurogenic patterns, however, were between the ages of 2 months and 3.5 years, suggesting premature EMG abnormalities in our group^{6,24}.

Nemalin bodies or rods are a sign of NM, and the best technique for detecting them is the MGT staining of biopsy sections^{1,2,5,11}. With other staining or histochemical techniques, such as ATPase staining, the results are often not distinct or can show a mild negative image. Nemaline bodies are distributed randomly along the muscle fiber, but have a tendency to be attached below the sarcolemma (subsarcolemmal location), or appear as difused spots in the interior of the fiber (intermyofibrillar location). Mixed patterns can also occur. Intranuclear rods have been described in severe neonatal and adult forms^{26,30}. Rods were identified in less than 50% of muscle fibers in six of our

patients and more than 50% in two of our patients via MGT staining. All cases showed a mixed location pattern, regardless of the NM clinical form.

The predominance of type I fibers is a common feature in NM, and some patients can have exclusively type I fibers or poor differentiation between fibers^{5,12-14}. Infrequently, there is a focal increase in acid phosphatase activity in fibers, which is associated with cases with a fast evolution. This suggests a degenerative process, inducing lysosomal enzyme activation and cellular destruction^{13,14}. Our cases showed a predominance of type I fibers and increased acid phosphatase activity, regardless the NM clinical form.

The analysis of nebulin protein expression using immunohistochemical began in the last decade and there are descriptions of normal and abnormal expression patterns in patients with NM, mainly for the typical form¹⁵⁻¹⁷. Nebulin acts as a molecular ruler for thin filaments and is important for the assembly and integration of Z discs with the sarcomere^{15,17}. In our study, the typical form of NM was associated with abnormal nebulin expression, as were the severe and intermediate congenital forms, which is similar to reports in other studies¹⁵⁻¹⁷. For desmin protein expression, the presence of areas of accumulation in muscle fibers is a pathological finding and may represent immaturity or a muscle fiber regeneration process. Van der Ven described strongly positive desmin expression in cases of severe congenital NM, while in mild cases, there was not a distinct expression pattern¹⁸. In our study, both the severe congenital form and the typical and intermediate congenital forms showed strongly positive desmin expression in muscle fibers. Fast myosin expression was absent in two patients, while positive expression with a mosaic pattern was observed in the majority of patients.

Six NM-related genes, all encoding components of the thin filament of the sarcomere, have been described. Studies of several families with severe, intermediate and typical NM forms have identified mutations in β -tropomyosin (TPM3) and beta-tropomyosin¹⁹⁻²¹. A recent study described an Australian family with a mutation in the TPM3 gene²². Several mutations were identified in the nebulin (NEB) gene due to small deletions or insertions^{22,23}. Mutations in the α -actin gene (ACTA1) may be related to congenital fiber type disproportion^{24,25}, and have been reported to be exclusively associated with intranuclear rods²⁶. Troponin T gene (TNNT1) mutations were described in an isolated group of old order Amish NM patients^{4,24}. A sixth gene, CFL2, encoding the actin-binding protein muscle cofilin-2, was reported in two siblings with congenital myopathy²⁷. Our patients could have had any of these genetic alterations. Our patients had abnormal nebulin expression by immunohistochemistry suggesting mutations in nebulin. Mutations in CFL2, ACTA1 and TNNT1 are less probable to occur in our patients because we had only one case of

the congenital form of NM, and there were no cases with intranuclear rods or Amish patients. Unfortunately, mutations in many of these genes do not predict the severity or prognosis of the disease and do not determine patterns of inheritance³¹.

In the present study, we focused on protein expression analysis in patients with NM and concluded that regardless of the NM clinical form, nebulin may have heterogeneous or absent protein expression, and fast myosin protein expression may be positive with a mosaic pattern in the majority of patients. In addition, the desmin protein, which is related to the maturity level of the muscle, may show normal or abnormal expression in NM patients.

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