Rod Distribution and Muscle Fiber Type Modification in the Progression of Nemaline Myopathy

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Rod Distribution and Muscle Fiber Type Modification in the Progression of Nemaline Myopathy

ABSTRACT

Nemaline myopathy is a structural congenital myopathy associated with the presence of rodlike structures inside the muscle fibers and type I predominance. It may be caused by mutations in at least five genes: slow α-tropomyosin 3 (chromosome 1q22-23), nebulin (chromosome 2q21.1-q22), actin (chromosome 1q42), tropomyosin 2 (chromosome 9p13), and troponin T1 (chromosome 19q13.4). The effect of these mutations in the expression of the protein and the mechanism of rod formation is still under investigation. We analyzed the possibility of progressive alterations with time and disease evolution, such as transformation of type I to type II fiber and rod pattern and distribution in muscle fibers from patients with nemaline myopathy, through a morphometric and immunohistochemical analysis of different muscle protein isoforms. A tendency of diffuse rods to be organized in the subsarcolemmal region was observed in two patients who were submitted to subsequent biopsies after 10 and 13 years. Additionally, we observed the expression of type II protein isoforms in type I fibers and a higher proportion of type II fibers in the younger patient of a pair of affected sibs, giving further support to the hypothesis of progressive conversion of type II to type I fibers in nemaline myopathy. (J. Child Neurol 2003;18:235-240).

Nemaline myopathy, with an estimated incidence of 2 per 100,000 live births, is a structural congenital myopathy associated with the presence of rodlike structures (nemaline bodies) inside the muscle fibers.1–2

Clinically, nemaline myopathy is characterized by the presence of hypotonia as well as proximal and facial weakness associated with skeletal deformities. According to the degree of muscle weakness, severity, and age at onset and based on correlations from the international database on nemaline myopathy, five forms were clinically defined by the European Neuromuscular Center (ENMC) International Consortium on Nemaline Myopathy.3

Nemaline myopathy can show both autosomal dominant and autosomal recessive inheritance patterns, and mutation in at least five genes has been identified in affected patients. Nemaline myopathy type 1 is caused by mutations in the slow α-tropomyosin 3 gene (TPM3, at chromosome 1q22-23),4,5 nemaline myopathy type 2 by mutations in the nebulin gene (at chromosome 2q21.1-q22),6 nemaline myopathy type 3 by mutations in the actin gene (at chromosome 1q42),7 nemaline myopathy type 4 by mutations in the tropomyosin 2 gene (at chromosome 9p13),8 and nemaline myopathy type 5 by mutations in the troponin T1 gene (at chromosome 19q13.4), which was recently described in Amish patients with an estimated incidence of 1 per 500 live births.9 The effect of these mutations in the protein expression and the mechanism of rod formation is still under investigation.

The “rods” or nemaline bodies, which are pathologic hallmarks of this disorder, can appear within the fibers as compact subsarcolemmal forms, fine diffuse structures, or both.10–12 Usually, the rods are present in the type I fibers, and the proportion of fibers containing rods can also vary among individuals.2,12,14 Progressive alterations related to time of the disease have been proposed, such as augmentation of rod number in muscle fibers and rod migration from the subsarcolemmal to the intermyofibrillar position.12

Another important histopathologic feature frequently observed in nemaline myopathy is the type I predominance, which can be total in some cases. Based on observation from consecutive muscle biopsies done in the same patient, a substitution of type II fiber to type I fiber has been suggested to occur.2,13,12

The expression of different protein isoforms leads to distinct muscle fiber phenotype with different mechanical and energetic properties, which is one of the main determinants of muscle performance in vivo. The histologic identification of three main human muscle fibers, type I, IIA, and IIX (previously called IIB), is based on two different histochemical approaches: adenosine triphosphatase (ATPase) activity and oxidative enzyme activity. More recently, the identification of myosin heavy chain isoforms allowed demonstration that the histochemical ATPase is based on the presence of specific myosin heavy chain isoforms (see revision in Bottinelli and Reggiani15).

The α-actins are cytoskeletal proteins belonging to the spectrin gene superfamily. α-Actin isoforms have multiple roles in different cell types. The skeletal, cardiac, and smooth muscle isoforms are localized in the Z-disk and in analogous dense bodies, where they help to anchor the myofibrillar actin filaments.16 Three human α-actin genes have been identified: one nonmuscle cytoskeletal isoform (ACTN1), mapped at chromosome 1q42-24, and two muscle-specific isoforms, ACTN2 and ACTN3, mapped, respectively, to chromosomes 1 (1q42-43) and 11 (11q13-14).16 ACTN2 is present in all types of muscle fibers, whereas ACTN3 is present only in type II fibers, 100% of type IIB and 50% of type IIA.17

The main objective of this study was to analyze the possible progressive alterations in muscle biopsies from patients with nemaline myopathy related to fiber type differentiation, rod frequency, and their pattern of distribution within muscle fibers. The type of fiber differentiation was studied through immunohistochemical reactions using ACTN3 and fast myosin antibodies and compared with ATPase reactions. The opportunity to study subsequent biopsies done in two patients after 10 and 13 years allowed us to verify in a longitudinal analysis the tendency of diffuse rods to be organized in the subsarcolemmal region. Additionally, the expression of type II proteins isoforms in type I fibers and a higher proportion of type II fibers in the younger patient of a pair of affected sibs with mutation in the nebulin gene gave further support to the temporal conversion of type II to type I fibers in nemaline myopathy.

Patients and Method

A total of 15 patients (from 14 unrelated families) with a diagnosis of nemaline myopathy were included in this investigation. The diagnosis was based on clinical examination, course of the disease, family history, serum creatine kinase levels, electromyography, and muscle biopsy. The clinical
classification was done according to data proposed by the ENMC International Consortium on Nemaline Myopathy in 1999. Partial DNA analysis has been described previously and included the deletion of 2bp in exon 173 of the nebulin gene in 2 sisters and exclusion of the Met9Arg in the TPM3 gene in 11 patients.16

Muscle samples were obtained from biceps or deltoid biopsies, frozen in liquid nitrogen immediately after removal, and stored at −70°C, staying there until time of use. Routine histologic and histochemical procedures were done, with staining for hematoxylin and eosin (H&E), modified Gomori trichrome, reduced nicotinamide adenine dinucleotide (NADH), ATPase 9.4 and 4.3, and acid and alkaline phosphatase.21 In two patients, a second muscle biopsy was done after 10 and 13 years in the same muscle (biceps in the first and deltoid in the second). Partial data from the first biopsy were obtained from slides stained for modified Gomori trichrome and NADH. In addition, data from muscle biopsy of two sisters with a mutation in nebulin gene, aged 10 months and 3 years,18 were analyzed.

The fiber type was determined by counting 1000 fibers from each patient in ATPase 9.4 and 4.3 reactions and by calculating the percentage of type I and type II fibers. A predominance of type I fibers was to be considered when more than 55% of type I fibers were present.10

For the analysis of the proportion of fibers with rods, 500 to 1000 fibers of each patient were analyzed and the percentage of fibers with and without rods was calculated. In addition, a classification of the rods and their pattern of distribution was done as described below: subsarcolemmal: when they were localized in a compact manner close to the fiber membrane; diffuse: when several small rods were distributed across the whole fiber; and mixed: both patterns together.18

Immunohistochemical staining of frozen sections was done through double labeling reactions,19 using a rabbit polyclonal antibody for α-actinin 3 diluted 1 to 100 and a mouse monoclonal antibody for fast myosin (Sigma, St. Louis). As second antibodies, antirabbit and antimouse IgG antibodies, both FITC and CY3 conjugated, were used. The sections were analyzed under a Zeiss Axiophot microscope with epifluorescence or confocal microscopy.

Results

Histologic and Histochemical Analysis
Among the 15 patients studied, the age at biopsy ranged from 10 months to 29 years. According to clinical classification, 3 patients had the intermediate form (patients 2, 9, and 15), whereas all of the other 12 patients had the typical form (Table 1). Family history suggested an autosomal recessive inheritance in 4 patients (from 3 pedigrees), whereas the remaining patients were sporadic cases.

All patients showed rods in muscle fibers, with a proportion between 100% and 40% of fibers (see Table 1). The distribution of the rods inside the fibers showed the following patterns: it was predominantly diffuse in two patients (patients 8 and 10) and subsarcolemmal in eight patients. In five patients, diffuse rods were also present in association with the subsarcolemmal rods, which were classified as mixed rods.

Statistical analysis showed no significant correlation between age at biopsy and proportion of fibers containing rods (r = .09, P = .74). However, considering the pattern of rods and age, we observed that among the five patients over 10 years old, four of them showed a predominance of subsarcolemmal rods, whereas among the five patients showing predominance of mixed rods, four were less than 5 years old.

Predominance of type I fibers was observed in all patients and varied between 100% (in 10 patients) and 60% (patient 1) (Table 2).

No correlation was observed between the age at biopsy and type I predominance (r = .39, P = .15). However, a partial predominance of type I fibers was detected mainly in younger patients, whereas in older patients, the predominance of type I fiber was total.

Immunohistochemical Analysis
The proportion of positive fibers for ACTN3 and fast myosin was analyzed and compared with the ATPase reaction (see Table 2).

Among the 10 patients who showed 100% of type I fiber on ATPase reaction, 7 showed a variable number of positive fibers on ACTN3 and/or fast myosin reactions (Figure 1). One patient showed a similar proportion of positive fibers with the two antibodies (case 12). Patient 5 showed a higher proportion of positive fast myosin fibers, whereas 5 patients presented a higher proportion of positive ACTN3 fibers (cases 4, 9, 11, 13, and 14).

Among the five patients who showed type II fibers on ATPase reaction, two revealed a similar proportion of type II fibers on the immunohistochemical reactions. One patient (case 7) showed 13%
of type II fiber on fast myosin reaction and a lack of reaction with ACTN3 antibody. Patient 2 showed a higher proportion of positive fibers on ACTN3 reaction when compared with the fast myosin reaction.

### Table 2. Immunohistochemical Analysis of Fiber Typing in 15 Patients With Nemaline Myopathy

<table>
<thead>
<tr>
<th>Case</th>
<th>Age at Biopsy</th>
<th>Type I/II Fibers (%)</th>
<th>% of Type II Fibers in ATPase Reaction</th>
<th>% of Type II Fibers in Fast Myosin Reaction</th>
<th>% of Type II Fibers in ACTN3 Reaction</th>
<th>Positive ACTN3 Fibers That Are Negative in Fast Myosin Reaction</th>
<th>ACTN3 Positive Internal Structures in Type I Fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 mo</td>
<td>60/40</td>
<td>40</td>
<td>40</td>
<td>36</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>9 yr</td>
<td>70/30</td>
<td>30</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>2 yr</td>
<td>80/20</td>
<td>20</td>
<td>20</td>
<td>38</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3 yr</td>
<td>90/10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>5 yr</td>
<td>93/7</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>3 yr</td>
<td>100/0</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>4 yr</td>
<td>100/0</td>
<td>0</td>
<td>22</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>4 yr</td>
<td>100/0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>6 yr</td>
<td>100/0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>8 yr</td>
<td>100/0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>13 yr</td>
<td>100/0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>14 yr</td>
<td>100/0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>18 yr</td>
<td>100/0</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>26 yr</td>
<td>100/0</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>29 yr</td>
<td>100/0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

+ = present; – = absent; ATPase = adenosine triphosphatase.

Figure 1. Double immunohistochemical analysis using antibodies for fast myosin (MY-FAST) and α-actinin 3 (ACTN3) and the superposition of both reactions (MERGE) in three patients (original magnification ×400).
Among the 15 patients studied, 9 showed some positive fibers in ACTN3 reaction, which were negative on fast myosin reaction (see Table 2 and Figure 1). Ten patients showed fibers with an intermediate pattern of reaction on ACTN3 reaction: strongly ACTN3-positive structures in type I fibers.

The two sisters (cases 1 and 3) showed a similar proportion of fibers containing rods. Both of them showed large subsarcolemmal rods; however, in the younger one, there was 65% of the fibers showing subsarcolemmal rods associated with diffuse rods (mixed), whereas in the older one, these mixed rods were observed in only 10% of fibers (see Table 2 and Figure 2).

The two sisters showed the presence of type II fibers on ATPase reaction with a higher proportion in the younger one (40%) when compared with the older sister (10%) (see Figure 2). The immunohistochemical analysis for ACTN3 and fast myosin showed a similar proportion of type II fibers when compared with the ATPase reaction. The two sisters showed positive ACTN3 fibers, which were negative for fast myosin reaction and type I fibers with ACTN3-positive internal structures.

Two patients were submitted to a second muscle biopsy. In patient 12, his first biopsy was done at 18 months old and the second at 14 years old. The comparative histopathologic study showed a high frequency of fibers with mixed rods (both subsarcolemmal and diffuse) and the presence of type I predominance in the first biopsy.20 The second biopsy showed a predominance of subsarcolemmal rods and a total predominance of type I fibers (Figure 2).

Patient 13 also showed a higher proportion of fibers with mixed rods in the first biopsy and a predominance of subsarcolemmal rods in the second one 10 years later (Figure 2). A total predominance of type I fibers was observed in the second biopsy. No fiber type data were available from the first biopsy.

**Discussion**

Congenital myopathies are characterized by a variability in the clinical course, which may range from nonprogressive to a slow or a fast progressive course. A large clinical follow-up study has shown a deterioration in muscle strength and respiratory function in about 16% of the patients with congenital myopathies. In addition, a sudden death of some patients, caused by respiratory or cardiac failures at the age of 20 years, has been described, even in benign congenital forms.21

The association of the progressive clinical worsening with modifications in the histologic pattern has been discussed. In patients with nemaline myopathy, a pathologic follow-up study done in 1988 detected some progressive alterations, such as an increase in the amount of rods in relation to age, migration of the rods from the subsarcolemmal to the intermyofibrillar position, and an increase in the endomysial fat or fibrosis.22 In patients with nemaline myopathy with a rapidly progressive course, a focal myofibrillar degeneration associated with an increase in lysosomal enzyme activity has been described.22 Therefore, these data provide evidence that progressive histopathologic alterations can occur in congenital nemaline myopathy.

In this study, we did not observe any correlation between age at biopsy and proportion of fibers containing rods. In addition, in the two sisters with the same genetic defect, aged 10 months and 3 years old, a similar proportion of fibers containing rods was observed. Therefore, new rods are apparently not formed with the evolution of the disease. Alternatively, some differences were observed in the pattern of rod distribution inside the fibers. In younger patients, there was a predominance of mixed rods, whereas in older patients, the subsarcolemmal rods were predominant. As for the two sisters, we observed a predominance of mixed rods in the younger sister, whereas subsarcolemmal rods were predominant in the oldest one. The same tendency was observed in the two patients submitted to two consecutive biopsies. Although previous data have indicated a migration of rods to the myofibrillar position related to age in some patients,22 our results suggest a tendency of a higher proportion of diffuse rods mixed with subsarcolemmal rods in younger patients, which become more organized, forming subsarcolemmal rods in the older ones. It is important to point out that the distribution of the nemaline rods can vary substantially from one individual to another and even between different muscles of the same patient. Our biopsies samples, however, were always...
collected from the same muscle (biceps or deltoid), allowing us to do a more reliable comparison.

On the other hand, a clear pattern of predominance of small broadly distributed diffuse rods was present in two patients, aged 9 and 6 years old, which suggests a specific pattern of distribution that could be a consequence of a specific genetic defect.

The mechanism of rod formation is still under investigation. Recent studies on a transgenic mouse line with the Met9Arg mutation (TPM3 gene) have shown that mutant tropomyosin expression did not result in immediate formation of rods, suggesting that rod formation is secondary to contractile dysfunction. Therefore, mutations in the nebulin gene could lead to abnormalities in the structure of the protein, causing its distorted connection to the Z band, which would result in sarcomere dysfunction and subsequent rod formation. Similarly, mutations in other genes coding for sarcomeric proteins could be associated with contractile dysfunction of the sarcomere and rod appearance. The genetic defect could be related to higher or smaller sarcomere instability, which could affect the rod size and distribution. Rods have been also described in other unrelated conditions, such as human immunodeficiency virus (HIV) myopathy, central core disease, and mitochondrial myopathies, corroborating the hypothesis of the presence of rods as a secondary phenomenon to pathologies that are associated with abnormalities in muscle contractile apparatus.

The classification of muscle fibers is based on the histochemical analysis for ATPase activity and on the evaluation of myosin heavy chain isoforms. In addition, there is evidence that the expression of the isoforms of other myofibrillar proteins, such as the myosin light chains, tropomyosin, components of the troponin complex, and Z-line and M-line proteins, are also coordinated with that of myosin heavy chain isoforms. During prenatal development, there are differences of myosin isoform expression among fibers: fast myoblasts showed fetal, embryonic, and slow myosin, which will be changed by fast myosin in mature fibers; slow myoblast showed embryonic and slow myosin, with the latter persisting in adult slow fibers. These differences occur during the myoblast period, when the innervation is multiple, suggesting the presence of a myogenic regulation. Later, the adult phenotype appears (identified by ATPase staining and enzyme histochemistry), and around 1 year postnatally, all fibers are fully differentiated. Finally, during postnatal life, the fiber phenotype can still be altered owing to variations in neural discharge patterns, mechanical load, and hormonal stimulation.

In nemaline myopathy, as in other congenital myopathies, the proportion of fiber types is deviated and a type I predominance is frequently observed. Studies done on consecutive biopsies suggested a transformation of fiber type based on the disappearance of type II fibers with age. In our study, fiber type data from two families confirmed this possibility: the analysis of the two sisters showed a high proportion of type II fibers in the younger, and a consecutive study in patient B showed the presence of type II fibers in the first biopsy and a total type I predominance in the second biopsy 10 years later. Although fiber typing can also vary between muscles in the same patients, in these two cases, the comparison was done in exactly the same muscles.

The fiber type differentiation was traditionally demonstrated by enzyme histochemistry (ATPase reaction). More recently, this analysis has been done through immunohistochemistry, using antibodies against myosin heavy chains and α-actinin isoforms. Abnormalities in fiber type differentiation based on protein expression have been described in patients with nemaline myopathy, such as the coexpression of fast and slow myosin in the same muscle fiber and the presence of ACTN3 in type I fiber. Similar results were observed in our study, with the expression of ACTN3 and fast myosin isoforms in patients with a total predominance of type I fibers. In addition, some alterations in the expression of muscle isoforms, such as the presence of ACTN3 in fibers negative for fast myosin (in 9 patients) and the presence of ACTN3-positive structures inside type I fibers (in 10 patients), were also observed. Therefore, the identification of expression of type II isoforms in type I fibers suggests intermediate stages of fibers type transformation related to time following the evolution of the disease. However, an alternative explanation to be considered is that the isoform expression pattern might primarily deviate from the normal ones in this disease.

In one patient from our study (case 7), the presence of type II fibers was detected through the ATPase and fast myosin reactions. However, a lack of reaction with the ACTN3 was observed. It has recently been shown that a deficiency of α-actinin 3 may occur in several forms of muscular dystrophies. In addition, DNA studies of the α-actinin gene showed the presence of polymorphism in 16% of the world population, which leads to total deficiency of the protein in the muscle. It is possible, therefore, that our patient has this polymorphism. Two other patients showed fast myosin-positive fibers, which were negative for the ACTN3 reaction (cases 1 and 5), which could represent type IIa fibers because only 50% of them revealed ACTN3 expression.

In conclusion, our results in patients with nemaline myopathy also show progressive histopathologic alterations through time. Among them, possible conversion of fiber type I to II is relevant. Classically, neural influence and mechanical stimulation have been the main determinants of fiber phenotype alterations; however, a myogenic regulation may be considered. In view of the primary defects of nemaline myopathy, which consisted of mutations in muscle protein genes (tropomyosin 2 and 3, nebulin, actin, and troponin T1), it is possible that alterations in those proteins could have some influence on the fiber type differentiation processes. Additional studies on the physiopathologic mechanisms of congenital myopathies will give further information about the functions and interrelations of these muscle proteins.

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