Nebulin expression in patients with nemaline myopathy

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Abstract

Nemaline myopathy is a structural congenital myopathy which may show both autosomal dominant and autosomal recessive inheritance patterns. Mutations in three different genes have been identified as the cause of nemaline myopathy: the gene for slow α-tropomyosin 3 (TPM3) at 1q22–23, the nebulin gene (NEB) at 2q21.1–q22, and the actin gene (ACTA1) at 1q42. The typical autosomal recessive form appears to be the most common one and is caused by mutations in the nebulin gene. We have studied the pattern of nebulin labeling, in patients with the typical congenital form (ten patients), the severe congenital form (two patients) or the mild, childhood-onset form (one patient), using antibodies against three different domains of nebulin. A qualitative and quantitative nebulin analysis in muscle tissue showed the presence of nebulin in myofibers from all patients. Some differences relating to the rod structure were observed. The majority of the largest subsarcolemmal rods were not labeled with the N2 nebulin antibody (I-band epitope) and showed an indistinct pattern with the two antibodies directed to the Z-band portion of nebulin (epitopes M176–181 and serine-rich domain). Diffuse rods were not revealed using the three antibodies. A discordant pattern of nebulin N2 epitope labeling was found in two affected sisters with a mutation in the nebulin gene, suggesting that modifications in nebulin distribution inside the rods might occur with the progression of the disease. Western blot analysis showed no direct correlation with immunofluorescence data. In nine patients, the band had a molecular weight comparable to the normal control, while in one patient, it was detected with a higher molecular weight. Our results suggest that presence/absence of specific nebulin Z-band epitopes in rod structures is variable and could depend on the degree of rod organization.

Keywords: Nemaline myopathy; Nebulin; Congenital myopathy

1. Introduction

Nemaline myopathy (NM) is a non-progressive muscular disorder associated with the presence of rod-like structures (nemaline bodies) inside the muscle fibers and with an estimated incidence of 2 per 100,000 live births [1].

The presence of ‘rods’ or nemaline bodies in the muscle fibers is the pathological hallmark of this disorder. Within the fibers, the rods can be present as compact sub-sarcomeritic forms, as fine diffuse structures, or both [1–5]. Usually, the rods are present in the type I fibers, that are often predominant. But the proportion of fibers containing rods can also vary among individuals and among different types of muscles [1,4–9].

Clinically, NM 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, the ENMC Nemaline Consortium at its workshop in June 1999 [10] suggested the following classification: (1) severe congenital form, with contractures and no spontaneous movements or no respiration at birth; (2) the typical form, with onset in early childhood with weakness especially pronounced in the facial, bulbar and respiratory muscles and in the neck flexors; proximal more than distal weakness, milestones delayed but reached, slow or non-progressive course; (3) intermediate congenital form, with...
infantile onset, breathing and moving at birth, but later in childhood unable to achieve respiratory independence, sitting or walking, or use of wheelchair before the age of 11 years; (4) mild childhood or juvenile-onset, with no facial weakness and no foot drop; (5) adult form with onset of symptoms in adult age; (6) other forms, associated with cardiomyopathy, ophthalmoplegia, unusual distribution of weakness or intranuclear nemaline bodies [1,4–21].

Genetically, NM may show both autosomal dominant and autosomal recessive inheritance patterns [1,5,6,10,21–23]. The dominant and recessive forms are clinically and histologically similar.

Up to now, mutations in three different genes have been identified as the cause of NM: The first, NEM1 form, mapped to 1q22–23, was identified by Laing et al. [22]. A missense point mutation (Met9Arg) in codon 9 of the slow α-tropomyosin 3 (TPM3) was found in a large Australian autosomal dominant kindred with late childhood onset [24]. Subsequently, a homozygous missense mutation in the same gene was found in a patient with presumed recessive nemaline myopathy and a severe phenotype [25].

The second, NEM2 form, was identified by Wallgren-Pettersson et al. through linkage analysis, at 2q21.1–q22, in seven European families [23,26,27]. The candidate gene in this region was the nebulin gene, and recently, pathogenic mutations were found [28]. To date, 11 different mutations have been identified in 11 families of different ethnic origins. The patients are compound heterozygous for the mutations in six of the families, and homozygous in five. The majority of the mutations are small deletions or insertions causing frameshifts, or base substitutions causing stop codons. The clinical spectrum in these patients showed that nine had the typical form, three had the severe form, two had the mild form and one the intermediate form [10].

Very recently, mutations in a third gene, the actin gene at 1q42, were found in patients with either congenital myopathy with excess of thin filaments (n = 3), severe NM (n = 11) or mild NM (n = 4). Both autosomal dominant and autosomal recessive inheritance were described for this form [29].

The direct correlation between the different mutations and the expression of the proteins in the muscle is still under investigation for these three genes.

The main objective of this study was to analyze the localization and quantity of nebulin in muscle biopsies from patients with NM, using antibodies for three different domains of nebulin, and to correlate both with the clinical variability.

2. Patients and methods

A total of 13 patients (from 12 unrelated families) with a diagnosis of NM were included in this investigation.

The diagnosis was based on clinical examination, course of the disease (using the protocol of the ENMC International Nemaline Myopathy Consortium [10], family history, serum creatine-kinase levels, electromyography and muscle biopsy.

Muscle samples were obtained from biceps or deltoid biopsies, frozen in liquid nitrogen immediately after removal and stored at −70°C until use. Routine histological and histochemical procedures were done, with staining for HE, modified Gomori trichrome, NADH, ATPase 9.4, 4.3 and acid and alkaline phosphatase [9].

The fiber typing 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. We considered a predominance of type I fibers to be present when there were more than 75% of fiber type I.

For the analysis of the proportion of fibers with rods, 800–1000 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: (a) subsarcolemmal, when they were localized in a compact manner close to the fiber membrane; (b) diffuse, when several small rods were distributed across the whole fiber.

The subsarcolemmal rods were classified in large and small, based on the measurement and calculation of their relative size inside the fibers: The diameter and area of 100 fibers and the diameter of their respective rods were measured, using a specific software (KS300, Zeiss). We also calculated the proportional area that the rods occupy inside the fiber and the number and proportion of fibers with a large rod (when it occupied more than 11% of the fiber) and a small rod (less than 10% of the fiber) were calculated. In addition, the average of rod size for each patient was calculated (Table 2).

Immunohistochemical staining of frozen sections were done through single and double labeling reactions [30], using a rabbit polyclonal antibody for α-actinin 2 (kindly provided by Dr A. Beggs) diluted 1/100, as a marker for rod structure. Three different antibodies for nebulin were used: a mouse monoclonal antibody directed to an I-band epitope near the N2 line region, diluted 1/200 (Sigma [31]). In addition, two rabbit polyclonal antibodies were used which had been raised to the expressed serine-rich domain, and to the M176–181 domains. Both polyclonal antibodies were diluted 1/10 [32,33]. As second antibodies, anti-rabbit and anti-mouse IgG antibodies both FITC and CY3 conjugated were used.

For the analysis of nebulin distribution in muscle fibers, the following data were considered: the pattern of labeling in the entire fiber and the pattern of labeling of the rod, identified through labeling with an antibody for ACTN2. According to the intensity of labeling inside the rods, they were considered positive (strong labeling), equal (indistinguishable from the remaining fiber cross-sectional area), or negative (no labeling at all).

Western blot was done with 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and transfer at 150
V for 1 h [32]. The blots were incubated and revealed first with antibody for nebulin (Sigma), and subsequently, with antibody against ACTN2. The incubations with primary antibodies were done overnight, and the detection was done using alkaline phosphatase-conjugated secondary antibody.

To determine the relative protein content of the samples, a relative quantitative densitometric analysis was done for each patient, comparing nebulin quantity to ACTN2 band concentration. In addition, this proportion was assessed in normal control muscle, at different concentrations of muscle extract (100, 50, 25%).

In 11 patients, the TPM3 gene was analyzed for presence or absence of the Met9Arg mutation (cases 1, 3, 4–13) according to the methodology described by Laing et al. [24]. Screening for mutations in the nebulin gene is ongoing in patients 2, 7 and 8 [28], and 26 exons, starting from the 3′ end encoding the Z-disc part of this enormous protein, have been analyzed to date.

3. Results

3.1. Clinical evaluation

Data on clinical assessment are shown in Table 1.

Among the 13 patients (from 12 unrelated families), two showed the severe congenital form (patients 3 and 4), ten the typical form (patients 1, 2, 5–12) and one the mild childhood or juvenile-onset form (patient 13). The age at biopsy varied between 10 months and 28 years. The patients with the severe congenital form have respiratory insufficiency and still require mechanical ventilation. Patient 3 is unable to walk and patient 4 walks with assistance.

Among the patients classified with the typical form, all are ambulant. Three (1, 2, 5) suffered some complications during the first year of life. Patient 1 had swallowing difficulties, necessitating a gastrostomy. Patients 2 and 5 presented with recurrent pneumonia. Their muscle power improved with time, and they became able to walk with assistance or independently. Bone deformities (such as kyphoscoliosis, high arched palate, pes cavus and thoracic deformities) were observed in nine patients, and facial weakness was present in eleven patients. Patient 12 died suddenly from an unknown cause.

Family history suggested an autosomal recessive inheritance in three patients (from two pedigrees), while the remaining patients were sporadic cases.

3.2. DNA analysis

DNA analysis did not reveal the Met9Arg mutation in the 11 patients in whom the study was done.

Screening for mutations in about 14% of the nebulin gene in patients 2, 7 and 8 detected a mutation in one allele in both affected sisters: a 2 bp deletion in exon 173, which causes a stop at codon 6154, and was not detected in 284 control chromosomes.

3.3. Histological and histochemical analysis

Predominance of type I fibers was observed in almost all patients and varied between 100% (in eight patients) and 60% (patient 7) (Table 2).

All patients showed rods in muscle fibers, with a proportion between 100 and 41% of fibers (Table 2).

The distribution of the rods inside the fibers showed the following patterns: it was predominantly diffuse in two patients (patients 5 and 12) and sub-sarcolemmal in 11 patients. The subsarcolemmal rods were classified as being predominantly large in seven patients (1, 2, 4, 7–9, 11) and small in four patients (3, 6, 10, 13). In seven of these

Table 1
Clinical data

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age of onset</th>
<th>Age at biopsy</th>
<th>Clinical form</th>
<th>Respiratory insufficiency</th>
<th>Gastrostomy</th>
<th>Facial dysmorphism</th>
<th>Bone deformities</th>
<th>Inheritance</th>
<th>Maximal motor ability</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Birth</td>
<td>14 years</td>
<td>TF</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>S</td>
<td>Walks assisted</td>
</tr>
<tr>
<td>2</td>
<td>Birth</td>
<td>28 years</td>
<td>TF</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>S</td>
<td>Walks assisted</td>
</tr>
<tr>
<td>3</td>
<td>Birth</td>
<td>2 years</td>
<td>SCF</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>S</td>
<td>Non-ambulant</td>
</tr>
<tr>
<td>4</td>
<td>Birth</td>
<td>8 years</td>
<td>SCF</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>S</td>
<td>Walks assisted</td>
</tr>
<tr>
<td>5</td>
<td>Birth</td>
<td>6 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>S</td>
<td>Walks</td>
</tr>
<tr>
<td>6</td>
<td>Birth</td>
<td>3 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>S</td>
<td>Walks</td>
</tr>
<tr>
<td>7</td>
<td>Birth</td>
<td>10 months</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>AR</td>
<td>Walks</td>
</tr>
<tr>
<td>8</td>
<td>Birth</td>
<td>3 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>AR</td>
<td>Walks</td>
</tr>
<tr>
<td>9</td>
<td>7–8 months</td>
<td>13 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>S</td>
<td>Walks</td>
</tr>
<tr>
<td>10</td>
<td>Birth</td>
<td>5 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>AR?</td>
<td>Walks</td>
</tr>
<tr>
<td>11</td>
<td>4 months</td>
<td>4 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>S</td>
<td>Walks</td>
</tr>
<tr>
<td>12</td>
<td>Birth</td>
<td>9 years</td>
<td>TF</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>S</td>
<td>Walks</td>
</tr>
<tr>
<td>13</td>
<td>?</td>
<td>25 years</td>
<td>MCF</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>S</td>
<td>Walks</td>
</tr>
</tbody>
</table>

a SCF, severe congenital form; TF, typical form; MCF, mild childhood form.
b S, sporadic; AR, autosomal recessive.
c Patients 7 and 8 are siblings.
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>% Type I/I fibers</th>
<th>% Of fibers with rods</th>
<th>Predominant rod pattern</th>
<th>Rod size proportion of fibers (% occupied in the fiber)</th>
<th>Antibody N2: pattern of rod labeling on nebulin stain</th>
<th>Antibody M101-102</th>
<th>Antibody M176–181</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>98</td>
<td>Subsarcolemmal large</td>
<td>32 68 14.2</td>
<td>0 2% 98% N</td>
<td>= V</td>
<td>= V</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>97</td>
<td>Subsarcolemmal large + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43 57 12.6</td>
<td>2% – 98% I</td>
<td>= =</td>
<td>= V</td>
</tr>
<tr>
<td>3</td>
<td>80/20</td>
<td>90</td>
<td>Subsarcolemmal small + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53 47 10.9</td>
<td>Present&lt;sup&gt;c&lt;/sup&gt;</td>
<td>present N</td>
<td>= Mosaic</td>
</tr>
<tr>
<td>4</td>
<td>1000</td>
<td>100</td>
<td>Subsarcolemmal large</td>
<td>8 92 20.3</td>
<td>0 30% 70% N</td>
<td>= x</td>
<td>= =</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>100</td>
<td>Diffuse</td>
<td></td>
<td>No Reveal faulty</td>
<td>N</td>
<td>= =</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>92</td>
<td>Subsarcolemmal small + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71 29 8.9</td>
<td>42% 30% 28% N</td>
<td>= =</td>
<td>= =</td>
</tr>
<tr>
<td>7</td>
<td>60/40</td>
<td>98</td>
<td>Subsarcolemmal large + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 95 27.9</td>
<td>Present&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Present N</td>
<td>= Mosaic</td>
</tr>
<tr>
<td>8</td>
<td>90/10</td>
<td>100</td>
<td>Subsarcolemmal large + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5 90.5 20.9</td>
<td>0 20% 80% N</td>
<td>= V</td>
<td>= V</td>
</tr>
<tr>
<td>9</td>
<td>1000</td>
<td>76</td>
<td>Subsarcolemmal small and large</td>
<td>46 54 20.7</td>
<td>2% faulty 51% faulty 47% faulty N</td>
<td>= Faulty</td>
<td>= Faulty</td>
</tr>
<tr>
<td>10</td>
<td>95/5</td>
<td>44</td>
<td>Subsarcolemmal small</td>
<td>94 6 5.8</td>
<td>77% 23% 0 N</td>
<td>= =</td>
<td>= =</td>
</tr>
<tr>
<td>11</td>
<td>1000</td>
<td>99.5</td>
<td>Subsarcolemmal large + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 58 13.3</td>
<td>4% 57% faulty 39% N</td>
<td>= =</td>
<td>= =</td>
</tr>
<tr>
<td>12</td>
<td>70/30</td>
<td>40.6</td>
<td>Diffuse</td>
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<td>No Reveal faulty</td>
<td>N</td>
<td>= Mosaic</td>
</tr>
<tr>
<td>13</td>
<td>100</td>
<td>95</td>
<td>Subsarcolemmal small + diffuse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95 5 5.0</td>
<td>0 100% 0 N</td>
<td>= =</td>
<td>= =</td>
</tr>
</tbody>
</table>

<sup>a</sup> WB, Western blot; N, as normal control; I, higher than normal control; V, rods not positive but structurally visible; faulty, small areas with no labeling inside the fibers.

<sup>b</sup> More than 30% of the fibers containing diffuse rods also.

<sup>c</sup> Analysis impaired due to the small size of the fiber.
11 patients, diffuse rods were also present in association with the subsarcolemmal rods.

3.4. Protein studies

3.4.1. Immunohistochemical analysis in fiber cross sections

In control muscles, all the three nebulin antibodies showed the typical cross-striation pattern of sarcomeric proteins throughout the fibers. Antibody M176–181 also showed the mosaic pattern of type I/II fibers, that was not observed with the antibodies directed to the N2-line and Z-line epitopes, with slow type I fibers more intensely labeled than fast type II fibers.

None of the patients showed a complete absence of nebulin in muscle fibers with the three antibodies for nebulin. In three patients (5, 9, 10), a faulty pattern was observed, with small areas with no labeling inside the fibers. It was more intense in patient 9, using the N2 antibody (Fig. 1). In patient 9 only, this faulty pattern was also observed with the other two nebulin antibodies.

A fiber-typing mosaic pattern with antibody M176–181 was observed in three patients, which was compatible with the proportion of type I/II on ATPase staining.

3.4.2. Rod labeling pattern

The proportion of subsarcolemmal rods that were revealed using antibodies for the ACTN2 and N2 domains of nebulin was similar in all patients, taking into account both negative and positive images of these structures.

Immunohistochemical analysis of the rod structure, using the N2 nebulin antibody, showed that a positive labeling pattern was predominant in the small rods, while a negative staining of nebulin was observed in the majority of the largest sub-sarcolemmal rods. (Table 2 and Fig. 1). With antibodies from the serine-rich domain (M101–102) and M176–181, the rods were equally labeled, although the structure of the rods was visible in some patients (Fig. 2). The diffuse rods were not revealed by the nebulin reaction with any of the three antibodies.

3.5. Western blot analysis

Western blot (WB) analysis was possible only with antibody N2. In three patients there was not enough protein in

Fig. 1. Examples of double immunofluorescence patterns for nebulin (N2 antibody) and ACTN2 staining of the rods, showing a positive nebulin pattern (+), a negative pattern (−), a pattern indistinguishable from that seen in the areas of the fiber not occupied by rods (=) and a faulty pattern in the cross-sectional area of the fiber.

Fig. 2. Schematic structure of the nebulin gene and immunofluorescence analysis of muscle biopsy from patient 1, showing negatively labeled rods with antibody from the N2 line (left), and indistinguishable labeling with antibodies from the M176–181 (middle) and serine-rich domain (right) antibodies. Asterisks denote identifying rods.
the muscle extract for analysis (patients 3, 4, 13) while in the remaining ten patients the presence of nebulin was confirmed. In nine of these 10 patients, the band had a molecular weight comparable to that of the normal control, while in one patient (patient 2), a band with a higher molecular weight was detected (Fig. 3). The relative proportion of nebulin to ACTN2 in normal muscle varied between 37 and 52%, and did not depend on the different muscle protein concentrations. In the 13 patients, nebulin quantification on blots was compromised in 7 patients due to muscle extract degradation. In the remaining six, there was apparently no correlation with the immunohistochemical data: patients with undetectable nebulin labeling of diffuse rods (patients 5 and 12) as well as those with negative large subsarcolemmal rod labeling on IF (patient 8; Table 2 and Fig. 3) showed strong bands on WB.

3.6. Intrafamilial correlation

Histochemical study in the two sisters showed a higher proportion of type 2 fibers in the younger one (40%) when compared with the older sister (10%). Both of them, however, showed the same predominant pattern of large subsarcolemmal rods.

Immunofluorescence (IF) study with the N2 nebulin antibody showed different patterns of labeling: the younger, 10-month-old girl (patient 7) showed some large sub-sarcolemmal nebulin-deficient rods, other positively-labeled rods and a global mosaic-like nebulin pattern on muscle fibers. Her older sister (aged 3 years) (patient 8) showed a homogeneous pattern of labeling of fibers, with predominance of large sub-sarcolemmal rods that were deficient for nebulin (Fig. 4). With the two other nebulin antibodies, the rod structures were indistinct inside the fiber. A mosaic of fiber typing was observed with the M176–181 antibody in the younger sister (Fig. 4).

4. Discussion

Nebulin is a giant protein (600–800 kDa) found in skeletal muscles, which accounts for 3–4% of the total myofibrillar proteins [33]. The C-terminal region of nebulin is located at the Z lines, whereas its N-terminal end is at the pointed end of the thin filament [31,33–35]. Nebulin acts as a molecular ruler for the thin filament and is important for the assembly and integration of Z discs with the sarcomere [28]. Mutations in the nebulin gene were recently found in patients affected by the typical congenital form of NM [28]. However, the correlation between the mutations and the expression of the protein in the muscle is still under investigation.

According to the clinical course, ten patients from the present study could be candidates for the nebulin-related typical NEM2 form [1,6,10]. All presented with clinical features compatible with the typical congenital form although not all had facial weakness or skeletal deformities. At muscle biopsy, in addition to the presence of rods, a predominance of type I fibers was observed. Analysis of the proportion between type I and type II fibers in the two affected sisters showed a more preserved mosaic pattern (60/40 type I/II) in the younger 10-month-old girl when compared with her 3-year-old sister (90/10 type I/II). These data are in accordance with previous pathological studies which suggested a progressive transformation of type II to type I fibers [4,8].

4.1. Nebulin in muscle fibers

The IF analysis for nebulin using three antibodies against different domains of this protein showed a positive pattern in the muscle fibers of all patients. In addition, the presence of a normal molecular weight nebulin band, using the N2 nebulin antibody, in nine out of ten patients, suggests that the primary defect in these patients would neither be related
to a total deficiency of the protein nor to an alteration in protein size.

Comparing patients with the severe congenital form with those who had the typical form, no difference in nebulin pattern appears to occur.

The observation of a higher molecular weight band in one patient, a finding to our knowledge not reported previously, suggests a mutation associated with the expression of a larger protein, e.g. an insertion. There is another possibility also, relating to differential splicing of the nebulin gene. Several differentially expressed exons have been identified in the region encoding the Z-disc part of nebulin [10,28,34]. In addition, five novel nebulin exons have been detected in the mRNA of one Australian patient with a severe form of NM. The physiological role of these exons remains unknown [10]. Therefore, although the finding of a nebulin band with a higher molecular weight in one patient warrants further studies to confirm a primary nebulin mutation, other possible explanations relate to these newly described phenomena in this gene are alternative splicing and additional exons. On the other hand, we cannot discard the possibility of altered migration due to artifact in this muscle extract.

Previous immunohistochemical studies of nebulin, performed in patients from families in which linkage results were compatible with linkage to the 2q locus, did not show any staining differences between muscles of patients and normal controls [1,6,36]. In 1999, Pelin et al. [28] performed immunocytochemical reactions using antibodies specific for the C-terminal SH3 domain of nebulin and for nebulin single repeats M176–181, and correlated the results with the different nebulin mutations found in the same patients. In this analysis, all patients showed presence of nebulin, and no specific differences were observed in the pattern of staining within the rod structures. All the cases showed normal labeling with the M176–181 antibody when compared with controls. Only one patient, with a mutation in M185, showed no reaction with the SH3 antibody and an uneven labeling in the larger fibers, with the M176–181 antibody [28]. In the present study, we used three different antibodies for nebulin and observed that the pattern of the entire fiber reaction was abnormal in one patient (patient 9), with all three antibodies. This abnormality consisted of small areas with no reaction for nebulin. The possibility of frozen artifact could be excluded because these alterations were not observed on the histological staining and ACTN2 labeling. As this patient had a higher proportion of ‘moth-eaten’ fibers, this could be the cause of the pattern observed. Although more evident with the N2 nebulin antibody, the faulty pattern was not correlated to the absence of a specific nebulin epitope. It is possible, however, that it could represent a particular abnormality of NM, and in this case, our patient could be a candidate for a mutation in the nebulin gene.

A fiber-type mosaic pattern has been described in muscle from normal controls, with the antibody M176–181 for nebulin, with slow type I fibers more intensely labeled than fast type II fibers [28]. We observed a mosaic pattern with the M176–181 antibody in three patients, who had a higher proportion of type II fibers (patients 3, 7, 12). The N2 and serine-rich domain antibodies did not show a mosaic pattern in any of the normal or pathological muscles studied. With the N2 nebulin antibody a mosaic-like nebulin pattern was observed in the same 10-month-old patient, which could be a reflection of the positively and negatively labeled rods observed in this patient. This was surprising, mainly because it was not seen in her older sister, suggesting an alteration related to the earliest stages of the disease.

4.2. Nebulin distribution in the rods

We analyzed nebulin labeling using antibodies against three different domains of the protein. Two of them, antibodies M176–181 and serine-rich domain (M101–102), were against epitopes localized in the Z bands, and the third N2 nebulin antibody, against an epitope localized near to N-terminal region [31], within the I band of the sarcomere.

Immunofluorescence reaction with the two Z-disc antibodies showed that all the rods, independently of their size,
were labeled with the same intensity as the remaining fiber. The presence of some of the largest, dense subsarcolemmal rods could be identified because of differences in the striation pattern. However, with the nebulin antibody from the I band, it was observed that many of the largest subsarcolemmal rods were predominantly negative, while small sarcolemmal rods were mostly positive. Additionally, the diffuse rods were not detectable by nebulin antibody labeling. Therefore, the presence of specific epitopes of nebulin in these structures may be related to the size and the degree of organization of the rods. It is tempting to suggest that the large subsarcolemmal rod would present a demarcated structure, with a compaction of proteins predominantly from the Z disc, while the diffuse and small rods, positively labeled with all antibodies, would be formed by a wider mixture of proteins.

Nebulin analysis in the rods from the two sisters gives further support to the hypothesis that as the disease progresses, modifications in nebulin presence and/or distribution inside these structures could occur.

The mechanism of rod formation is still unknown. A primary defect in the nebulin gene could lead to abnormalities in the nebulin structure causing an altered connection of this protein at the Z disc and, as a consequence, sarcomere instability and rod formation. Perhaps through similar mechanisms, mutations in other genes for the proteins of the thin filament and Z disc, such as the tropomyosin and actin genes, also lead to rod formation. Our results suggest that the presence/absence of the nebulin in rod structures is variable and could depend on the degree organization and compaction of rods. Alternatively, the immunohistochemical visualization of nebulin at rod structures could be impaired by masking of the epitopes by other sarcomeric proteins related to the Z disc, as already suggested for other proteins [37,38].

It is known that WB studies are unable to detect small alterations in protein quantity. However, the lack of correlation between WB data and IF pattern of nebulin in the rods provides further evidences for the hypothesis that the presence or absence of nebulin in the rod structures is not a reflection of quantitative alterations but a consequence of reorganization of proteins.

In summary, based on IF and WB analysis, our studies show the presence of nebulin in muscle fibers of all the patients studied with congenital NM. Abnormalities of nebulin distribution within the rods could be a consequence of reorganization of sarcomeric proteins. WB analysis may in some cases help to reveal a nebulin abnormality.

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