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Clinical and genetic heterogeneity in nemaline myopathy – a disease of skeletal muscle thin filaments

Despina Sanoudou and Alan H. Beggs

The term nemaline myopathy (NM) encompasses a heterogeneous group of disorders of primary skeletal muscle weakness characterized by the presence of nemaline rods in muscles of affected individuals. Disease severity is variable and unpredictable, with prognosis ranging from neonatal death to almost normal motor function. Recent advances in the identification of NM disease genes demonstrate that NM is a disease of the skeletal muscle sarcomere and, in particular, of the thin filaments. These findings are starting to alter the approach that neurologists and geneticists take to diagnosing and counseling patients with NM, and could lead to insights into specific directed therapies in the future.

Nemaline myopathy (NM) is a slowly- or non-progressive neuromuscular disorder characterised by muscle weakness and the presence of rod-shaped structures [NEMALINE RODS (see Glossary)] in affected muscle fibers (reviewed in Ref. 1). NM was first described in 1963 by Conen *et al.*[2] and Shy *et al.*[3] and its name reflects the perceived thread-like appearance of the rod bodies (nema being the Greek word for thread). Although a relatively rare disease, it is the most common of the non-dystrophic CONGENITAL MYOPATHIES, occurring worldwide with an estimated incidence of 0.02 per 1000 live births[4]. Many cases are sporadic, but some exhibit either autosomal recessive or dominant patterns of inheritance. As with most other congenital myopathies, NM is pathologically defined on the basis of structural abnormalities of the muscle fibres, which can be visualized after staining of muscle biopsy sections by histochemical or electron microscopic methods. Recent progress in the identification of five different NM genes has shown that, despite a great degree of both clinical and genetic heterogeneity, the common pathological findings are related to the fact that each NM gene encodes a known component of skeletal muscle sarcomeric THIN FILAMENTS.

Clinical description
The nemaline myopathies are defined by primary proximal muscle weakness associated with a myopathic muscle biopsy, characterized by the presence of nemaline rods, and the absence of clinical or pathological findings diagnostic of other disorders. The wide range of clinical presentations represents a continuum from neonatal-lethal forms to late onset slowly progressive weakness[5]. However, to facilitate further study, including potential phenotype–genotype correlation, some categorization has been attempted[6,7] (Table 1).

Typical NM
The typical (i.e. most common) form of NM is usually autosomal recessive and presents with congenital or infantile HYPOTONIA, weakness and often, feeding difficulties[4,8] (Table 1). In cases with profound weakness and hypotonia in the neonatal period, strength often improves with age, leading to delayed attainment of gross motor skills. Patients have a narrow face with a high arched palate, reflecting bulbar weakness, and a lean build, sometimes associated with muscular atrophy. Although fine motor activity is normal, gross motor activity is impaired. Proximal muscles are generally more affected than distal ones, although distal weakness, especially later in life, is not

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uncommon. The respiratory muscles are always involved, and nocturnal hypoxia and hypercarbia are a constant danger, even for patients with minimal skeletal muscle weakness. Joint hypermobility is commonly seen and joint deformities or contractures can occur congenitally or occasionally develop at later stages of the disease. The weakness is often static or only slowly progressive and many patients lead an active life. Some patients, however, will require a wheelchair following a progressive phase associated with the prepubertal growth spurt. Respiratory problems persist throughout the patient's life, and eventual death is often associated with respiratory

insufficiency[5]. As for all clinical forms, the central nervous system is unaffected, and intelligence and cardiac contractility are usually normal.

Mild NM
At the mildest end of the spectrum are patients with similar clinical and pathological findings to the typical cases, but with childhood onset of symptoms[5,9,10]. These cases are also often sporadic (or possibly, autosomal recessive) but occasionally can clearly be inherited as autosomal dominant traits. At later ages, these mild cases can be indistinguishable from typical cases with age of onset the only differentiating factor (Table 1).

Severe NM
Severe NM presents at birth with hypotonia and profound muscle weakness, particularly affecting the diaphragm and intercostal muscles, which results in severe respiratory insufficiency[5,8]. Patients have few spontaneous movements with difficulties swallowing and sucking. Occasionally, multiple contractures (ARTHROGRYPOSIS) are prominent features. Despite intensive neonatal care and ventilatory support, death from respiratory insufficiency or pneumonia often occurs in the first months of life. These cases can present significant prognostic dilemmas as some patients with these apparently severe initial presentations go on to survive with only minimal residual disabilities and retrospectively become classified in the

Table 1. Clinical classification and features of the nemaline myopathies[a]

| Category	Onset	Course	Contractures	Respiratory status	Motor milestones	Other findings	Genes involved
Severe	At birth	Often lethal in first year	Severe congenital or absent	Vent dependence from birth	No spontaneous movements at birth		*ACTA1, NEB,* other?
Intermediate	Birth → infancy	Slowly or non-progressive	Congenital or later onset or absent	Respiratory independence by 1 year but progressive deterioration	May never sit or walk or wheelchair-bound by 11 years		*ACTA1, NEB, TPM3,* other?
Typical	Birth → infancy	Slowly or non-progressive	Later onset or absent	Respiratory independence by infancy	Delayed, ambulation into adulthood		*ACTA1, NEB, TPM2,* other?
Mild	Childhood or juvenile	Slowly or non-progressive	No	Respiratory independence	Normal		*ACTA1, NEB, TPM2, TPM3,* other?
Adult onset	Adulthood	Progressive	No	Respiratory independence	Normal	Inflammatory changes, cardiomyopathy	Unknown
Amish	At birth	Progressive lethal by second year	Progressive	Death owing to respiratory failure	No antigravity movements	Neonatal tremors, pectus carinatum	*TNNT1*
Other forms	Variable	Variable	Variable	Variable	Variable	Occasional: cardiomyopathy, ophthalmoplegia, atypical distribution of weakness	Unknown

[a]Modified and updated from Ref. 6.
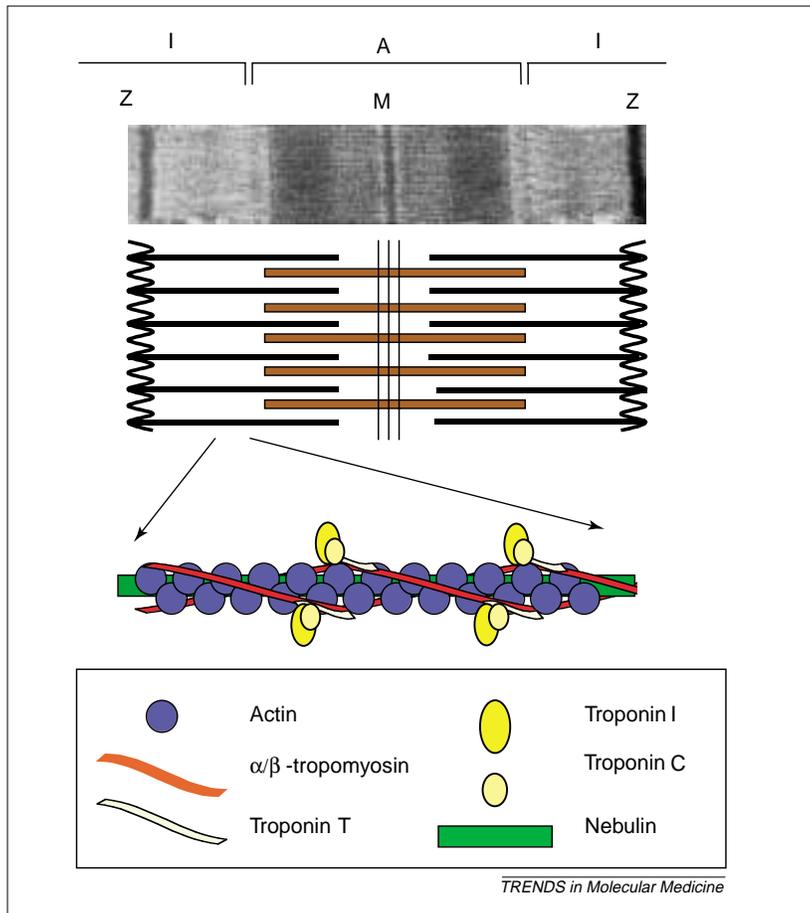
Fig. 1. Electron micrograph and schematic representation of the sarcomere and the localization of major thin filament proteins, many of which are abnormal in nemaline myopathy. The sarcomere is delimited by Z lines that bisect the actin thin-filament-containing I bands. A bands contain the myosin thick filaments centered about the M line.

'intermediate' or 'typical' groups. Recently, fetal akinesia sequence was shown to be one extreme presentation for NM (Ref. 11). As severe NM patients never survive to reproduce, these cases tend to be sporadic and are never seen in pedigrees with autosomal dominant inheritance, although some subset of families have multiple affected siblings, indicative of autosomal recessive inheritance[5,10].

Intermediate NM
Further prognostic confusion can arise in cases with either 'typical' presentations but progressive courses, or apparently 'severe' cases that survive infancy. These 'intermediate' cases are defined by survival past one year, the inability to sit and/or walk, or the loss of ambulation and progressive deterioration by age 11 years (Table 1).

Adult onset NM
There is also a progressive adult onset form that represents a separate, quite heterogeneous group of patients in terms of clinical presentation with onset in the third to sixth decade. In these cases with onset during this period, there is typically no family history and no preceding symptoms. In some cases,

progressive weakness is associated with inflammatory changes on muscle biopsy, suggesting that these cases might represent a distinctly different pathophysiological mechanism[12]. Others present with cardiomyopathy and only minimal skeletal muscle weakness[13].

Amish NM
Amish NM is a clinically distinct autosomal recessive form with neonatal onset and early-childhood lethality. To date, it has been described in only a single genetic isolate of related Old Order Amish families[14]. It presents at birth with hypotonia, contractures, and remarkably, tremors that typically subside over the first 2–3 months of life. Progressive weakness associated with severe pectus carinatum, muscle atrophy and contractures often leads to death owing to respiratory insufficiency in the second year of life.

Other forms of NM
A few remaining cases can be grouped into a heterogeneous category of 'other forms', including patients with atypical findings such as ophthalmoplegia or other unusual distributions of weakness and nemaline rods in skeletal muscle. It is presently unclear whether the molecular pathophysiology of these cases will justify categorizing them as variants of NM or as other diseases with associated rods.

Normal muscle structure
The contractile apparatus of skeletal muscle is composed of repeating units (SARCOMERES) that comprise ordered arrays of actin-containing thin filaments and myosin-containing thick filaments[15] (Fig. 1). Several other associated structural and regulatory proteins are present within the sarcomere to maintain the ordered myofibrillar array and coordinate contraction.

Each sarcomere is delimited by Z LINES, which are electron-dense bands, perpendicular to the myofibrils, that bisect the actin-containing I band and anchor the thin filaments[15]. Z lines are generally widest in type 1 (slow) and narrowest in type 2b (fast) fibers. The composition of Z lines is not well known, although it is evident that α-actinin is a major structural component, as is actin, the actin-capping protein CapZ, and several recently identified proteins including telethonin[16], myotilin[17] and myozenin[18]. The α-actinin forms diagonal cross-connections between the thin filaments, which are anchored via a network of interactions between α-actinin, actin, nebulin and other proteins.

The myosin-containing thick filaments (in the A bands) interdigitate with thin filaments that are made up of a double-stranded helix of globular actin monomers (e.g. F actin) associated with a single molecule of nebulin. At >770 kD, nebulin ranks as

one of the largest known proteins[19]. The central portion contains up to 185 tandem repeats of 35 residues, each of which probably binds a single actin monomer. The C-terminus is unique and is embedded in the Z lines. Extensive alternative splicing in the central repeat region yields different length nebulin molecules that are thought to regulate thin filament length through binding of the actin capping protein, tropomodulin, at the amino terminus of nebulin[20]. Along the length of the thin filaments, the tropomyosins and troponins together form a complex of proteins responsible for control of contraction by regulating the interactions of actin and myosin[15,21]. At rest, tropomyosin dimers lie along the actin filament in a potential myosin-binding site, sterically inhibiting myosin–actin interactions. Tropomyosin position and movement is controlled by the troponin complex consisting of three subunits, TN-I (inhibitory), TN-T (tropomyosin-binding), and TN-C (calcium-binding). When muscle is stimulated, intracellular calcium levels increase to a critical level, binding to TN-C and releasing the inhibitory effect of TN-I. Tropomyosin moves into the groove between actin helices, unmasking the myosin-binding sites and triggering the contraction cycle.

Muscle pathology in NM

Standard haematoxylin and eosin stained sections of skeletal muscle from patients with NM can appear normal, or can exhibit some fiber size variation, but staining of frozen sections by the Gomori trichrome method readily reveals the nemaline rods that are the hallmark of this disorder (Fig. 2). The rods vary from 1–7 μm in length and from 0.3–2 μm in width and stain dark red or purple contrasting with the pale blue-green myofibrils. They tend to cluster under the sarcolemmal membrane or around nuclei, and rarely can be intranuclear[22–24]. Although there has been some speculation that intranuclear rods might indicate a poor prognosis[23,24], there is a report of adult-onset NM with nuclear rods[22]. Moreover, the majority of severe cases do not contain intranuclear rods. The proportion of fibers containing rods can vary from less than 1% to virtually all fibers and does not correlate with the degree of muscle weakness, although more active muscles, such as diaphragm, can have greater numbers of rod-containing fibers. Histochemical stains for myosin ATPase reaction at different pH levels reveals the other common myopathic change in these muscles. Fiber type proportions and sizes can vary widely from the normal ratio of 1:1:1 of equal sized type 1:2a:2b fibers. Often, NM biopsies exhibit fiber type 1 predominance, and in extreme cases, fiber typing by the ATPase reaction is impossible owing to uniform reactivity of a pure population of type 1 fibers. Rods can be found equally in all fiber types or preferentially in either type 1 or type 2 fibers. Often,
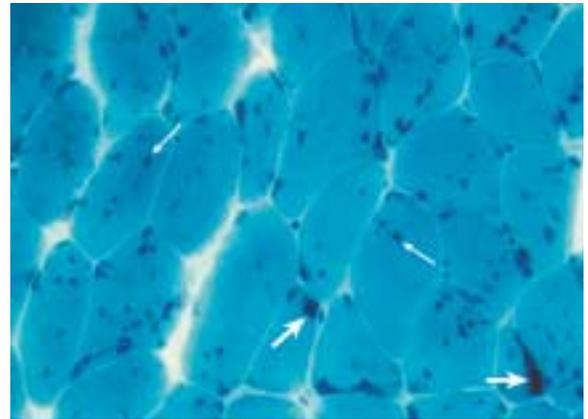

Fig. 2. Gomori trichrome-stained transverse section of skeletal muscle from a nemaline myopathy (NM) patient. Nemaline rods appear in virtually all fibers as darkly staining bodies distributed throughout the cytoplasm (small arrows) and as larger subsarcolemmal accumulations in occasional fibers (large arrows). Note that NM pathology is variable as biopsies from other cases do not necessarily have rods in all fibers, and rod-containing fibers can sometimes be significantly smaller than fibers without rods.

but not always, the rod-containing fibers are hypotrophic. Areas with extensive rod formation exhibit significant disruption and disorganization of sarcomeric structure, probably accounting for some degree of the associated weakness. Occasionally, muscle fibers are replaced by fat or fibrous tissue, but fiber necrosis and regeneration is rare. None of the variable pathological findings described above have been correlated with any particular clinical or genetic category of NM.

Electron microscopy reveals the definitive ultrastructure of the rods and can be used as an objective means to define these bodies (Fig. 3). The rods are electron dense and appear similar in composition to the Z lines from which they emanate[25]. They can appear as enlarged Z lines or elongated structures replacing thin filaments in a disorganized mass of sarcomeric components. Thickening and streaming of the Z lines is also sometimes seen, but these are not diagnostic features by themselves. Consistent with their appearance as extensions of Z lines, the rods are largely made up of α-actinin as well as several other Z line proteins[16–18,26]. Digestion with Ca^{2+}-activated protease removes the α-actinin, revealing an actin-containing thin filament backbone[27]. A three-dimensional reconstruction of rods has revealed adjacent actin filaments of opposite polarity cross-linked by probably α-actinin dimers[28], suggesting that rod formation might involve an inability to terminate or 'cap' the thin filament ends at Z lines.

It is important to note that nemaline rods are not pathognomonic for NM as they can be reproducibly induced to form in tenotomized rat muscle[29] or neostigmine myopathy[30] and are also rarely found in small numbers in normal or non-NM myopathic muscles as well as in HIV-associated myopathy[25,31]. In particular, they are also found in a subset of patients with central core disease caused by certain
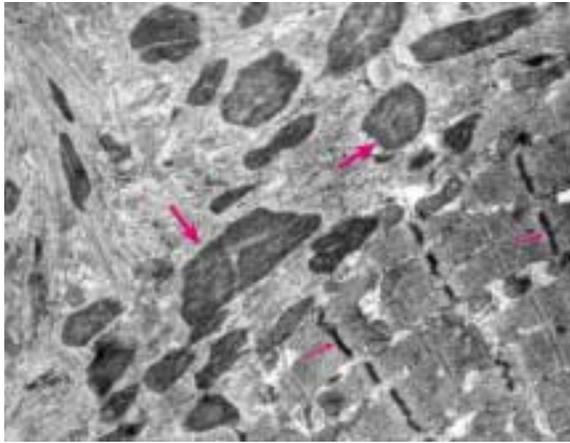
Fig. 3. Electron micrograph illustrating typical nemaline rods and disorganized myofibrillar apparatus in a patient with nemaline myopathy. An area of relatively normal muscle structure with normal Z lines (small arrows, bottom right) is seen next to a structurally disorganized region with multiple nemaline rods (large arrows) and disorganized thin filaments (top left).

ryanodine receptor gene mutations[32,33]. Thus, rod formation probably represents a common pathophysiological response of skeletal muscle to certain pathological situations. Understanding the mechanisms of (secondary) rod formation in these situations will probably provide important insights into the 'primary' or 'pure' nemaline myopathies.

Genetic basis of NM

As discussed below, NM is a disorder of sarcomeric thin filaments, making each thin filament or Z line protein a potential NM candidate gene. Indeed, all the NM genes identified to date encode known components of the sarcomeric thin filaments. Unfortunately however, mutations in many of these genes do not predict severity or prognosis of the clinical course (Table 1). Furthermore, this molecular variability extends to inheritance patterns, as at least some NM genes can cause sporadic cases, as well as autosomal dominant and autosomal recessive patterns of inheritance.

In 1992, linkage analysis of a large Australian family with a childhood onset autosomal dominant form of mild NM, allowed mapping of the first NM gene to chromosome 1q13–q25 (Ref. 9). Subsequent mapping and analysis of the slow α-tropomyosin gene (*TPM3*) in this family revealed a missense mutation, M9R (Ref. 34). This change was predicted to affect the N-terminal structure of the α-tropomyosin, possibly strengthening tropomyosin–actin interactions and leading to the formation of nemaline rods. Expression of this mutation *in vitro* did not result in rod formation[35], however, when introduced into a transgenic mouse line, *TPM3* M9R expression resulted in rod formation in all muscles and a late onset (5–6 months of age) skeletal muscle weakness[36]. These mice are an important resource for further studies on NM pathophysiology and the mechanisms of rod formation and weakness.

Two subsequent follow-up studies of 76 and 40 families, respectively have identified the second and third known instances of *TPM3* mutations. One homozygous nonsense mutation was identified in a consanguineous severe infantile case[37], and two additional recessive *TPM3* mutations were found in a compound heterozygous patient with intermediate NM (Ref. 38). The paternally-derived mutation eliminates the skeletal muscle-specific stop codon, resulting in production of an elongated protein containing an additional 56 amino acids. The maternal mutation disrupted a splice site at the same exon. Thus, NM *TPM3* mutations can have many effects on the protein and are rare, accounting for only 2–3% of NM cases (3/117). Unfortunately, no useful clinical predictions can be made, as these mutations have been associated with severe, intermediate and mild forms that can be either autosomal recessive or dominant.

Linkage analysis of seven European multiplex families with autosomal recessive typical NM suggested the presence of a second NM gene on chromosome 2q21.2–2q22 (Ref. 39). A further study of 22 families with autosomal recessive NM identified six mutations in the nebulin gene (*NEB*) at this locus[40]. All six mutations are predicted to cause truncation and loss of 25–80 kDa from the C-terminal end of the nebulin protein. However, immunofluorescence data show that extreme C-terminal epitopes are present in affected muscles, probably reflecting alternative splicing. Thus, the aberrant transcripts could be internally truncated and/or only a subset of nebulin transcripts might be aberrant, possibly resulting in pathology owing to lack of the normal physiological isoform diversity. Recent follow-up studies have identified additional recessive nebulin mutations in patients with wide-ranging phenotypes, although the majority appear to have the typical clinical presentations[6].

With the identification of NM mutations in the genes for slow α-tropomyosin and nebulin, a hypothesis emerged that NM was a disorder of sarcomeric thin filaments. This idea was further reinforced by the discovery that the skeletal muscle α-actin gene (*ACTA1*) could also be mutated in NM patients. Nowak *et al.*[10] started by screening *ACTA1* as a candidate gene in three patients with congenital myopathy and excess accumulation of thin filaments[41]. As several of these individuals also had occasional nemaline rods in their muscle, the study was extended to a series of 59 cases of severe neonatal, intermediate and milder forms of NM. In total, 15 mutations were identified in 14 families, including all three cases of excess thin filaments and 11 NM families whose affected members' phenotypes ranged from severe to typical. All the identified *ACTA1* mutations were heterozygous missense changes, most of which were autosomal dominant, or, in the genetically lethal severe cases, *de novo*. However, one family exhibited autosomal

recessive inheritance with two affected children who were compound heterozygotes for mutations inherited from each parent. Several additional *ACTA1* mutations have now been identified in NM patients with varying clinical presentations and inheritance patterns, further confirming the heterogeneous nature of the actin-related NMs (Refs 42,43).

Studies on a genetically isolated group of Old Order Amish NM patients revealed the involvement of yet another sarcomeric thin filament gene[14]. Linkage mapping in 33 related nuclear families identified a locus on chromosome 19q13.4 in the vicinity of the slow-fiber skeletal muscle troponin T gene (*TNNT1*). Mutation analysis revealed a homozygous stop codon mutation at amino acid 179 in exon 11 in all affected family members. The recessive nature of this mutation suggests that the loss of 83 C-terminal amino acids might destabilize the protein, resulting in deficiency of troponin T and possibly disruption of normal excitation–contraction coupling in affected muscle fibers. Whether or not this relates to the unique muscle tremors experienced by these patients remains to be determined. Preliminary RT-PCR-based mutation studies on a small group of non-Amish NM cases failed to identify additional *TNNT1* mutations, suggesting that these account for only a small proportion of cases at best (D. Wattanasirichaigoon and A.H. Beggs, unpublished).

Most recently, NM mutations have been found in another thin filament protein gene, that for β-tropomyosin (*TPM2*) on chromosome 9p13 (Ref. 44). Analysis of the *TPM2* gene in probands from 54 families revealed two dominant mutations in patients with typical clinical courses. Both were missense changes affecting highly conserved regions of *TPM2*. It is hypothesized that these mutations might affect the actin-binding properties of β-tropomyosin.

Several other genes have been studied for linkage to NM without conclusive results to date. Linkage analysis of ten patients from five families with autosomal recessive NM excluded the muscle specific α-actinin gene, *ACTN2* (on chromosome 1q42–q43)[45]. Linkage of NM to *TPM1* (on chromosome 15q22)[46] and *TPM4* (on chromosome 19)[47] was also ruled out in a small group of families in which *TPM3*, *NEB*, and *ACTA1* mutations were previously excluded[7]. However, these and other thin filament protein genes remain good candidates, as many NM cases are sporadic and not amenable to linkage analysis.

Diagnosis and treatment of NM

As with other congenital myopathies, the diagnosis of nemaline myopathy is primarily a pathological one that is made based on the observation of nemaline rods in Gomori trichrome-stained light, or electron microscopic, sections of skeletal muscle.

> **Outstanding questions**
>
> - What is the molecular mechanism of rod formation and how does this relate to muscle weakness?
> - How many additional NM genes exist? Will they all encode components of sarcomeric thin filaments?
> - Will any genotype–phenotype correlations emerge from analysis of larger patient groups?
> - What are the relationships between: (1) late-onset, rapidly progressive NM cases, (2) severe lethal cases of fetal akinesia, and (3) the more typical early onset, non-progressive ones?
> - Can we use our knowledge of the genetic defects in NM to devise specific therapies for this disorder?

Histological methods have been unsuccessful so far in differentiating between the different NM forms, however, there is preliminary evidence for several potential associations between morphological features and genotype or phenotype. Nuclear rods are not found in 'typical' cases but instead are associated with poor prognosis[23,24]. To date, prominent thin filament accumulations are only reported in some cases of actin mutations[10,41] and abnormal nebulin immunohistochemistry has been found in a subset of cases with nebulin gene mutations[40]. Confirmation of these findings awaits studies of larger series of cases. Mutation studies for NM are problematic owing to the large number of genes, the large size of the nebulin gene, and the fact that there could be yet unidentified NM genes, but improving technology for mutation detection should make molecular testing a reality in the foreseeable future.

At present there is no curative treatment for NM or other congenital myopathies. However, appropriate medical and orthopedic management can offer a better quality of life to many patients[48]. Supportive care in the neonatal period is essential as some 'typical' cases present with profound weakness and hypotonia indistinguishable from that in some severe cases. As with other myopathies and dystrophies, important factors influencing prognosis are respiratory capacity and the development of scoliosis. Careful monitoring of respiratory sufficiency, particularly during sleep, is crucial as some patients with minimal limb weakness could require nocturnal ventilation.

Conclusions

NM is a heterogeneous disorder whose etiology remained a mystery until the advent of molecular genetic studies. To date, several different, but overlapping, clinical forms of NM have been defined and defects in five distinct genes have been identified. However, there is substantial clinical

variation between patients with presumed genotypic homogeneity in families with both autosomal dominant and recessive inheritance. In addition, the genetic changes do not correlate well with clinical presentation. For example, mutations in both *TPM3* and *ACTA1* can be either autosomal dominant or recessive, and are associated with lethal congenital-onset as well as nonprogressive, later-onset forms of NM. Most probably, additional NM genes remain to be discovered and it will be interesting to see if all these also encode thin filament proteins. An important outstanding issue is our lack of knowledge about the molecular mechanisms underlying both rod formation and the associated weakness. The recent development of a mouse model[36], and advances in microarray-based gene expression analysis methodology could soon shed light on this and other relevant questions. Eventually, the acquired knowledge might improve diagnosis, treatment and prognosis for NM patients, while leading to new insights into Z line and thin filament structure and function.

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