Nemaline body myopathy caused by a novel mutation in Troponin T1 (TNNT1).

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Abstract

**Introduction:** Nemaline myopathy is a rare disorder characterized by skeletal muscle weakness of varying severity and onset, with the presence of nemaline rods on muscle biopsy. Congenital nemaline body myopathy due to mutations in *TNNT1* has hitherto only been described as a result of a single founder mutation in patients of Amish origin and in 2 other individuals with different recessive mutations.

**Methods:** Autozygosity mapping and Whole Exome Sequencing (WES) were applied after we identified 9 Palestinian patients from 7 unrelated families who suffer from nemaline myopathy.

**Results:** All patients were homozygous for a novel complex rearrangement of the *TNNT1* gene (c.574_577delinsTAGTGCTGT | NM_003283) leading to a C-terminal truncation of the protein (p.L203* | NP_003274.3). Their clinical course was remarkable for early respiratory failure and striking stiffness of the cervical spine.

**Discussion:** This report exemplifies the utility of combining Autozygosity mapping and WES and expands the phenotype associated with *TNNT1* mutations.
Introduction

Congenital nemaline body (NEM) myopathies are a heterogeneous group of hereditary myopathies characterized by skeletal muscle weakness and the presence of rod-like structures in skeletal muscle fibers which are best revealed by electron microscopy. Patients with NEM myopathy typically present with congenital proximal muscle weakness and hypotonia. Respiratory weakness is often a major concern, while intelligence is usually unimpaired.\(^1\)

So far, 10 autosomal genes with a multitude of mutations and recessive and dominant modes of inheritance have been identified as causative for NEM. These comprise *ACTA1, NEB, TPM2, TPM3, CFL2, KHLH40, KLHL41, KBTBD13, LMOD3*, and *TNNT1* with the first 2 being the most common.\(^1,2,3\) The genes known so far encode proteins that are associated with the thin filaments of the sarcomere except for *KBTBD13, KLHL40,* and *KLHL41* whose protein function is unknown.

Of note, mutations in several NEM genes have more than 1 clinical or histological presentation. For example, mutations in *ACTA1, NEB, TPM3,* and *KBTBD13* have been linked to NEM myopathies and other clinical spectra such as cap myopathies or distal myopathies.\(^1\)

While NEM myopathies as a group have an incidence of 1:50,000,\(^4\) those subtypes linked to *TNNT1*-mutations seem to be rare. Johnston *et al.* (2000) described a recessive NEM myopathy with lethal respiratory failure among Old Order Amish caused by a homozygous nonsense mutation, c.538G>T (p.E180*), introducing a stop codon in exon 11 of the *TNNT1* transcript.\(^5\) For a long time mutations in *TNNT1* were only known in Old Order Amish individuals. Recently, however, van der Pol *et al.* (2014) described a single patient with 2 novel compound heterozygous *TNNT1* mutations harboring a phenotype similar to that seen in the Amish.\(^6\) A further patient was described by Marra *et al.* (2014) whose clinical and histological features were compati-
ble with the diagnosis of NEM myopathy and carried a homozygous nonsense mutation, c.323C>G (p.S108*) in exon 9 of TNNT1.7

Together with Tropomyosin (Tm), Troponin (Tn) is involved in regulating electromechanical coupling between Ca\(^{++}\) influx from the sarcoplasmatic reticulum and subsequent muscle contraction. The thin multimeric Tm:Tn filaments run parallel to actin filaments and expose (in the presence of Ca\(^{++}\)) or occlude (in the absence of Ca\(^{++}\)) the actin binding sites for the myosin heads. Tn is composed of 3 subunits, the Ca\(^{++}\)-binding troponin C (TnC), the inhibitory troponin I (TnI), and the tropomyosin-binding Troponin T (TnT). The 3 subunits are encoded by a multi-gene family and multiple splice variants. Their composition varies depending on the tissue (e.g. skeletal versus cardiac muscle or developmental versus adult).8 The 16 exons of the slow skeletal TnT gene (TNNT1) are spliced into 4 main isoforms which are preferentially expressed in slow-contracting type I fibers.9 Fast type II fibers mainly express the fast skeletal TnT gene variants (TNNT3 or TNNI2),10 while TNNT2 is expressed mainly in cardiac muscle. The various isoforms differ with respect to amino acid composition, molecular weight, and regulation.9

Here we describe 7 Palestinian families with 9 children with congenital NEM caused by a novel homozygous rearrangement in TNNT1 (NEM5).

**Patients and Methods**

**Patients**

Informed consent was obtained from all parents and guardians according to the Declaration of Helsinki for all aspects of the study. The investigations were approved by the institutional review board of the Hadassah Medical Center.
A consanguineous index family presented with 2 children who were affected by congenital hypotonia and weakness. Clinical investigations were followed by autozygosity-mapping and whole exome sequencing (see below) that allowed us to identify a deleterious mutation of \textit{TNNT1}. Another 7 patients presented during the ensuing 4 years and included 2 sib pairs from different Palestinian families. Based on a similar clinical phenotype we suspected NEM myopathy and found the identical mutation. We collected clinical data concerning pedigrees, antenatal and birth history, clinical manifestation and chronology, body weight and growth, age at diagnosis, age at starting mechanical ventilation, and age at and cause of death. We reviewed the medical records concerning electrophysiological investigations, heart echocardiograms, serum Creatine Kinase (CK) levels and results of muscle histology. The living patients underwent physical examination to describe their phenotype in detail. Information about patients who had died prior to initiating this study was obtained from parents and hospital records.

\textbf{Molecular genetic investigations}

\textit{Autozygosity-mapping}: the Affymetrix Gene-Chip Human Mapping 250 K \textit{NspI} Array (Affymetrix, Santa Clara, CA) was used to genotype ~250,000 Single Nucleotide Polymorphism (SNP) markers throughout the genome in patients 1, 3, and 4 according to the manufacturer's protocol.

\textit{Whole exome sequencing}: Exon sequences were enriched in the DNA sample of patient 1 using SureSelect® v4 Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, California, USA). Sequencing was done on a HiSeq2000 machine (Illumina, San Diego, California, USA) as 100 bp paired-end reads. Data analysis including read alignment and variant calling was performed by the DNAnexus software (Palo Alto, California, USA) using the default parameters with the human genome assembly hg19 (GRCh37) as reference, as previously described.\textsuperscript{11}
Sanger sequencing, segregation analysis and determination of the carrier rate: Genotyping of the 9 patients and their first degree relatives for the TNNT1 splice-site mutation was performed by Sanger sequencing of exon 11 and the flanking intronic segments of TNNT1. The carrier rate for this mutation was determined among 100 healthy Palestinian individuals from the same geographic area as the patients that had been selected by convenience.

Results

Phenotype Analysis (Tables 1 and 2)

The prenatal history of all subjects was normal except for 3 subjects who had poor fetal movements in the last 8 weeks of pregnancy; a twin pregnancy with both affected fetuses was delivered prematurely at 33 weeks of gestation.

Birth weights were appropriate for gestational age. During the neonatal period transient tremor of the limbs and sometimes of the mouth were observed in 6 subjects over a few weeks. One subject suffered from bilateral hip dislocation and had to undergo surgical intervention.

At the ages 3-4 months most parents began to notice motor developmental delay and poor head movement that was associated with an apparently stiff neck. Passive motion was unrestricted during the first 3-4 months of life. Beyond that period all subjects suffered from progressive spinal rigidity with kyphosis, scoliosis, and limb contractures. All patients had severely delayed motor milestones. Two subjects were able to sit before the age 1 year, 1was able to stand, and another was able to stand, but subsequently lost this ability. All patients have preserved forearm and hand function with reduced strength and are only mobile in wheel chairs. Their voices are nasal, weak, and dysarthric. Dolichocephaly was observed along with facial weakness. Apparent
intelligence is preserved. All 4 living subjects are on assisted mechanical ventilation, 3 have a tracheostomy, and 1 is mechanically ventilated through a mask. The age of initiation of mechanical ventilation varied between 1.0 and 4.5 years. All subjects, except for 2, were fed through a gastrostomy that had to be established because of poor weight gain and recurrent aspiration. Routine laboratory investigations including CK were normal in all subjects. No cardiac involvement was seen on echocardiography.

Electromyography (EMG) was done in 7 subjects between ages 4 months and 3 years and revealed either normal findings in 3 (ages 15-17 months) or a mild inactive myopathic process in 3 (ages 4 and 6 months and 3 years). The 3-year-old patient with persistent tremor had an EMG suggestive of a neuromuscular junction disorder with increased neuromuscular jitter. The clinical features and the absence of a decrement on repetitive stimulation, however, excluded a myasthenic disorder. One subject (age 8 months) showed mildly active denervation with chronic re-innervation without accompanying pathological features to suggest neurogenic changes.

Muscle biopsies were performed in 7 subjects at the Hadassah Medical Center. Of the identical twins only 1 sibling underwent muscle biopsy. The available biopsies displayed non-specific myopathic-like changes, occasionally associated with ill-formed fiber type grouping. Nemaline rods were detected in 4 biopsies. Representative neuropathological features are shown in Figure 1.

Five patients died between the ages of 4 and 11 years from respiratory insufficiency. One sister of the diagnosed patients died at age 11 years with no genetic diagnosis at that time, however, clinical data are compatible with an NEM myopathy.

**Pedigree and autozygosity analysis**
The genome-wide SNP analysis in the samples of patients 1, 3, and 4 did not reveal a single homozygous region larger than 2 Mb with identical haplotype among them. Pedigrees were not connected despite the fact that patients originated from the same geographical region of the Palestinian West Bank.

**Whole exome analysis**

After mapping the reads and aligning them with the reference genome (DNA nexus platform), we called variants removing low depth (coverage below 8 fold), off-target, homozygous, X-linked, synonymous, and those present in more than 0.1% in the NHLBI Exome sequencing project or more than 1% of our in-house database. Of the 12 remaining variants, only 1 was clearly pathogenic, affected a known gene associated with congenital fiber-type disproportion and segregated with the disease phenotype in the families. This was a complex InDeletion in the *TNNT1* gene (chr19:55648472_55648475delinsACAGCACTA | c.574_577delinsTAGTGCTGT | NM_003283) which did not affect the nearby splice site (Figure 2), but its first 3 base pairs constituted an in-frame TAG stop codon (p.L203* | NP_003274.3). Instead of the wild type 251 amino acid-long protein, the resultant protein is predicted to lack its highly conserved C-terminus. A shared SNP haplotype encompassing 50 markers was noted for the 3 patients (1, 3, and 4) around the mutation site chr19:55.2-56.37 Mb (rs9797807-rs8104511) indicating an ancestral allele and founder mutation. *LMNA*, a gene in the differential diagnosis, was covered on average X65, and no mutation was found.

Homozygosity for the mutation was confirmed by Sanger sequencing in all patients, and heterozygosity was found in all their parents. PCR on cDNA of a patient homozygous for the c.574_577delinsTAGTGCTGT mutation verified the InDeletion on the mRNA level and excluded abnormal splicing (Figure 2).
Carrier rate in the Palestinian community.

No carriers of the mutation were detected from a convenience-selected sample of 100 Palestinians from the same geographical area. The disease allele hence cannot be a considered a frequent variant in the population.

Discussion

The patients share homozygosity for a novel mutation of \textit{TNNT1} predicted to cause frameshift of the protein. The phenotype is similar to that described in the Amish population affected by the p.Glu180* nonsense mutation, though several differences should be noted.

The Palestinian families were not knowingly related. The short shared haplotype segment of only 1.1 Mb enhances the assumption that we are dealing with an ancient founder haplotype, which, given its small size, might date back more than 100 generations.\textsuperscript{12}

The rigid spine found all of our patients was not described in the Amish patients and may lead clinicians to consider other disorders such as mutations in \textit{SEPN1} or \textit{LMNA} in the first place. The prominent tremor noted among all the Amish patients was present only transiently in our patients. There is apparent variability in the pathological features of this disease entity if it is compared to the Amish patients where nemaline rods were found ubiquitously. However, other key features of NEM myopathy were present in our patients and comprise: (1) small groups of atrophic myofibers, (2) abundant whorled-like myofibers, (3) focally increased endomysial fibrosis, and (4) type II fiber predominance with small type I fibers. Nemaline rods were detected in most biopsy specimens, but usually their presence was subtle and necessitated careful inspection of frozen sections, semithin sections, and electron microscopy.
Our patients suffered from respiratory insufficiency that ultimately led to their demise or necessitated long-term mechanical ventilation. The long-term outcome among the Amish patients was not reported. Pertinent with regard to the respiratory insufficiency is a study by Feng et al (2009) who investigated Tnnt1 knockout mice demonstrating a critical role of slow skeletal muscle troponin T (TnT) in diaphragm function. The authors showed that affected mice had diaphragm hypertrophy with an increase of fast fibers, thus reducing the fatigue tolerance of the diaphragm. The fatal outcome associated with NEM in our patients adds impetus to efforts to find a therapy which is presently not available. Two studies using L-tyrosine supplementation showed positive effects on muscle strength and mobility. One clinical open-label trial was performed in 5 individuals with NEM myopathy, and another study was done in mice with an Acta1 mutation (NEM3). In another recent report by de Winter et al (2013), a troponin activator (compound CK2066260) was used as a therapeutic substance to augment contractile protein function *in vitro* using muscle samples from patients with NEM myopathy who harbored NEB mutations.

Our report expands the phenotype associated with TNNT1 mutations and may lead clinicians to suspect NEM5 outside the Amish population.
List of abbreviations.

CK-Creatine Kinase

SNP- Single Nucleotide Polymorphism

NEM- Nemaline body

WES- Whole Exome Sequencing

Tm- Tropomyosin

Tn- Troponin

EMG- ElectroMyography

PCR- Polymerase Chain Reaction

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References


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<th>3</th>
<th>4</th>
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*Age at diagnosis* refers to diagnosis of myopathy.

N: normal, +: present, -: absent, PEG: percutaneous gastrostomy.
Table 2: Pathological features.

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Figure legends

**Figure 1.** Representative paraffin embedded and frozen sections show: (A) whorled-like myofibers (white arrow) and marked variation of fiber diameter, including small groups of atrophic fibers alongside hypertrophic fibers (desmin immunostain); (B) type II fiber predominance and fiber type disproportion with small type I fibers (arrows, ATPase 4.3 stain); (C) ill-formed fiber type grouping, with small groups of atrophic myofibers (encircled, H&E stain). Increased endomysial fibrosis is evident as well; Rod structures, consistent with nemaline rods were observed in 4 of 7 biopsy specimens, either on frozen sections (D) (modified Gomori trichrome stain, white arrows) and/or electron microscopy (E, F).

**Figure 2.** DNA sequence around the mutation site. (A) Sequence of a healthy control; the 4 nucleotides deleted in the patients are underlined; the arrow indicates the junction of exon/intron 10. (B) Sequence of a patient homozygous for the c.574_577delinsTAGTGCTGT mutation. The inserted nucleotides are underlined. *Note* the first 3 bases of the insert are an in-frame TAG termination codon; the arrow indicates the exon-intron junction. (C) cDNA of a healthy control demonstrating normal exon 10 / exon 11 splicing; the arrow indicates the exon/exon junction. (D) cDNA of a patient homozygous for the c.574_577delinsTAGTGCTGT mutation. The In-Deletion is marked.