CASE OF THE MONTH

NEB-RELATED CORE-ROD MYOPATHY WITH DISTINCT CLINICAL AND PATHOLOGICAL FEATURES

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ABSTRACT: Introduction: Mutations in the gene encoding nebulin (NEB) are known to cause several types of congenital myopathy including recessive nemaline myopathy and distal nebulin myopathy. Core-rod myopathy has recently been reported to be another type of NEB-related myopathy, and is pathologically characterized by the coexistence of cores and nemaline rods within muscle fibers. Methods: We describe 2 patients with core-rod myopathy who were analyzed genetically by whole exome sequencing and evaluated clinically and pathologically. Findings were compared with those of patients with the disease of other genetic causes. Results: Three NEB mutations were identified, 2 of which were novel. Mild clinical features, unusual patterns of muscle involvement, and atypical pathological findings were observed. Conclusions: We propose that the clinical and pathological spectrum of core-rod myopathy should be widened. A significant amount of residual nebulin expression is believed to contribute to the much milder phenotype exhibited by the patients we describe here.

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Nebulin is one of the giant proteins in sarcomeres. It spans the whole length of thin filaments and binds more than 200 actin monomers. In addition to maintaining the lengths of thin filaments, it is involved in cross-bridge cycling kinetics and in regulation of calcium sensitivity.1,2 Although mutations in the nebulin gene (NEB) have long been implicated in congenital myopathies, sequencing of NEB has been a challenge due to its huge size and the repetitive nature of its sequence.3 The recent advent of next generation sequencing (NGS) technology has facilitated access to large muscle genes like NEB and enabled interrogation of otherwise unsolved cases.

NEB mutations are mainly found in autosomal recessive nemaline myopathies, which usually present in typical congenital forms, but clinical severities vary from lethal neonatal-onset to mild adult-onset phenotypes.4,5 Recessive NEB mutations may also cause distal myopathies with or without nemaline rods in muscles.6,7 Another congenital myopathy with distinct pathological features of nemaline rods and cores has also been reported to be associated with NEB mutations in 2 patients, both of whom presented with progressive muscle weakness and respiratory insufficiency.3,8 This type of congenital myopathy, so-called “rod and core myopathy”, is also known to be associated with mutations of other genes, including ACTA1, CFL2, RYR1, KBTBD13, and TPM2.9–13

In our cases of distal myopathy with core-rod pathology, we successfully detected novel NEB mutations by whole exome sequencing. We describe in detail their clinical and pathological features and describe the patterns of protein expression affected by these mutations. This report expands the spectrum of phenotypes and histopathologies of NEB-associated myopathies.

PATIENTS AND METHODS

Patients. Two patients were included in this report based on their clinical features and the presence of nemaline rods and cores in their muscle biopsies. Informed consent was obtained from 1 patient and from the parents of the other. This study was approved by the ethical review board of Pusan National University Hospital.

Patient 1. A 43-year-old man presented with ankle weakness of 5 years duration. An elder brother was similarly affected. The patient had delayed motor developmental milestones and was unable to match his peers during athletic activities during school years. On presentation, ankle dorsiflexion and plantarflexion were graded as 2/5, and thigh and upper arm muscles were graded 4 to 5/5 on the Medical Research Council (MRC) scale. Asymmetrical calf atrophy was noted. Ankle...
contractures were prominent, but no other skeletal deformity, such as scoliosis, was observed. His cheek muscles were mildly affected, but extraocular movement was full without ptosis. A high-arched palate was not noted. Tendon stretch reflexes were absent in all limbs. Serum creatine kinase (CK) was within normal limits (103 IU/L, normal <217 IU/L). Muscle computed tomography (CT) scans showed greatest involvement of calf muscles, worse on the left side. Anterior tibialis muscles and a portion of posterior thigh muscles were second most affected (Figs. 1A and B). Pulmonary function testing was normal. During follow-up, ankle plantarflexion had worsened to MRC grade 1, but no worsening was recorded in any other muscle group. He maintained independent ambulation despite difficulty climbing and never complained of respiratory distress.

His affected sibling’s motor power was graded as MRC 2/5 for ankle dorsiflexion and 3/5 for plantarflexion, and tendon stretch reflexes were absent in all limbs. Calf muscles were asymmetrically atrophied, worse on the right. He was able to ambulate independently, and like his brother had no respiratory difficulty.

Patient 2. A 9-year-old boy presented with gait disturbance and a history of frequent falls since age 5. He had achieved normal motor developmental milestones, and his family history was not significant for any neuromuscular disease. At presentation, his height and weight were below the fifth percentile of the standard for his age. Muscle power for ankle dorsiflexion and plantarflexion were graded as 3/5, and thigh and upper limb muscles were grade 4+ to 5-/5. He had facial muscle weakness, but extraocular movements were normal. No high-arched palate was observed, but equinovarus deformity was noted. Serum CK was within normal limits (47 IU/L, normal: <217 IU/L). Muscle CT scans revealed slight involvement of calf muscles, while the anterior compartments of lower legs and thigh muscles were completely spared (Figs. 1C, D). Pulmonary function testing was normal, and scoliosis was not observed on X-ray. During 3 years of follow-up, he was able to run, but was slow and unstable.

Muscle Biopsy and Preparation for Light and Electron Microscopy. Muscle biopsy samples were obtained under local anesthesia from tibialis anterior muscles in both patients. Biopsied muscle specimens were flash-frozen and sectioned at 10 μm using a cryostat, and a series of histochemical stains, including hematoxylin and eosin, modified Gomori-trichrome, nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase (NADH-TR), cytochrome c oxidase (COX), and adenosine triphosphatase (ATPase), were performed. Muscle specimens were also fixed in 0.1 M cacodylate buffer containing 2% glutaraldehyde for electron microscopy, shaken in a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 M s-collidine for 2–3 h, and embedded in epoxy resin. Semi-thin 1-μm sections were stained with toluidine blue, and ultrathin 50 nm sections were stained with uranyl acetate and lead citrate.

Mutation Searches and Whole Exome Sequencing. Genomic DNA was extracted from the patient blood samples using the Wizard Genomic DNA purification kit (Promega, Madison, Wisconsin). Because the presence of nemaline rods was the most prominent pathologic muscle feature, genes associated with nemaline myopathy were initially analyzed by Sanger sequencing of ACTA1, CFL2, exon 147-183 of NEB, and exon 101 to 103 of RYR1, all of which are responsible for cores or core-rod pathologies.
Because the analysis for initially targeted genes returned negative findings, whole exome sequencing was performed. Exome capture was performed using the Illumina TruSeq Exome Enrichment Guide. Raw reads resulting from exome sequencing were aligned to the equCab2 reference genome of UCSC (University of California, Santa Cruz, CA) using BWA (bwa-0.5.9), default parameters, and the seed length 45 parameter. Aligned reads were processed, polymerase chain reaction duplicates were removed with SAMtoll (samtools-0.1.16), and further processed using CountCovariates, TableRecalibration, RealignerTarget-Creator, IndelRealigner step, and GATK (GenomeAnalysisTK-1.4). Pathogenicities of variants were predicted using Sorting Intolerant From Tolerant (SIFT), the Protein Variation Effect Analyzer (PROVEAN), PolyPhen2, Korean Personal Genome Project (KPGP) information, Online Mendelian Inheritance in Man (OMIM), and the Human Gene Mutation Database (HGMD). Candidate pathogenic mutations were confirmed using the classic Sanger method.

**Western Blot Analysis.** Proteins were extracted from the muscle samples of both patients and a normal subject. Quantified proteins from patients and controls were electrophoresed using 7.5% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Hercules, California) and transferred to PVDF at 8 V overnight. After blocking with 5% skim milk for 1 h, the blots were incubated with primary antibodies against nebulin (Myomedix Ltd., Neckargemünd, Germany), which recognize the N-terminal of the protein, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Bioworld, Louis Park, Minnesota) for 3 h. Horseradish peroxidase-conjugated ADI-SAB-300 and -100 (Enzo Life Science, Farmingdale, New York) were used as secondary antibodies for nebulin and GAPDH, respectively. Detection for relative quantitative analysis was performed using Multi Gauge version 2.1 (Fujiﬁlm, Tokyo).

**RESULTS**

**Morphology of Cores and Rods as Determined by Muscle Pathology.** The most prominent pathological findings in both patients were the muscle fibers with rods and cores. In patient 1, small areas with decreased oxidative enzyme activities were found on NADH-TR stain (Fig. 2A). Multiple core-like structures were sometimes present in a single fiber (multicores) mimicking a moth-eaten appearance, and most were poorly demarcated (Fig. 2B). Rod structures were frequently observed within muscle fibers on modified Gomori-trichrome stain. They were present in smaller-sized fibers and were mostly scattered through the sarcoplasm rather than being clustered in subsarcolemmal regions (Fig. 2C). In addition, a few ragged-red fibers were also noted (Fig. 2D), and they appeared as COX-negative fibers. In patient 2, core-like regions with deficient NADH-TR and COX enzyme activities were found exclusively in smaller-sized type 1 fibers (Figs. 2E and F). They were single and centrally located within muscle fibers. Rod structures in patient 2 were also scattered through the sarcoplasm (Fig. 2G), but some were clustered in subsarcolemmal areas (arrow in Fig. 2G). In serial sections, fibers with rods were associated with disrupted intermyofibrillar networks on NADH-TR stain, but core and rod-structures were observed in separate fibers. With myofibrillar ATPase stain, type 1 fiber predominance was observed in both patients (99% of type 1 fibers in patient 1 and 93% in patient 2). In patient 2, type 1 fibers were much smaller than type 2, producing an appearance of fiber type disproportion (Fig. 2H), although variation from fascicle to fascicle existed.

**Detection of Mutations by Whole Exome Sequencing.** Because no mutation was identified by Sanger sequencing of all coding exons of ACTA1 and CFL2, exons 101-103 of RYR1, and exons 147-183 of NEB, whole exome sequencing was performed. Sequence variants were filtered, and some further than 20 base pairs from exon-intron boundaries were removed. Based on considerations of clinical and myopathological conditions, 37 exon sequence variants in patient 1 and 38 in patient 2 were retained from RYR1, KBTBD13, and NEB. Of these variants, all of the RYR1 and KBTBD13 variants were synonymous or excluded by Sanger sequencing. By SIFT, PROVEAN, PolyPhen2, OMIM, and HGMD prediction, 3 of the NEB sequence variants were pathogenic; these variants were finally confirmed by Sanger sequencing. According to reference sequence NM_001271208.1 the identified mutations were as follows: c.3387delIC (p.Asp1129Aspfs*54), c.4617-4629del TTATAAACGAGAinsAAA (p.His1539Glnfs*12), and c.24579G>A (p.Ser8193Ser). Two of the frameshift mutations were novel and were found in each allele of patients 1 (c.3387delIC, p.Asp1129Aspfs*54) and 2 (c.4617-4629delTTATAAACGAGAinsAAA, p.His1539Glnfs*12). The splice site mutation of c.24579G>A (p.Ser8193Ser) was shared by both patients and has been reported previously in a patient with the typical
congenital phenotype of nemaline myopathy. The same NEB mutations identified in patient 1 were also detected from his affected brother by targeted sequencing. Parents of the patients were not examined due to death or divorce.

Nebulin Expression. Western blotting using antibodies targeting N-terminal and C-terminal epitopes of nebulin detected less protein in either patient than in normal controls (Fig. 3). Standardized immunoactivity against nebulin was 22.3% and 13.1% of normal control levels for the N-terminal epitope, and 40.4% and 31.6% for the C-terminal in patients 1 and 2, respectively.

DISCUSSION

Core-rod myopathy refers to a group of congenital myopathies in which cores and nemaline rods co-exist in muscle fibers. Although first reported in patients with RYR1 mutations, core-rod myopathy has also been reported in association with NEB, KBTBD13 (a member of the BTB/Kelch family), and TPM2 mutations. Before the reports of these genes as causative of core-rod myopathy, an ACTA1 mutation was reported to cause nemaline myopathy with cores, in which the core-like areas with unevenness of oxidative enzymes, as well as the presence of nemaline rods, were represented. Also, recessive mutations in CFL2, the gene encoding coflin-2 and a causative gene for nemaline myopathy type 6, have also been described to display focal loss of oxidative enzymes consistent with minicores. The pathological patterns of cores and rods in the disease appear to be variable and are probably partly dependent on the causative genes. Although nemaline rods in patients with RYR1 mutations are seen in various ways, cores are centrally located and well demarcated, as is seen in

**FIGURE 2.** Muscle pathology. (A) In patient 1, areas with deficient oxidative enzyme activity were frequently found on NADH stain. (B) In some fibers, multicores were present mimicking a moth-eaten appearance on NADH stain. (C) Rod structures were scattered within muscle fibers on modified Gomori-trichrome stain in patient 1. (D) In patient 1, a few ragged red fibers were observed on modified Gomori-trichrome. (E,F) In patient 2, core regions with deficient NADH and COX enzyme activities were found in smaller-sized type 1 fibers. (G) Most rod structures were scattered through the sarcoplasm, and some were clustered in subsarcolemmal regions (arrow) on modified Gomori-trichrome stain. (H) In patient 2, type 1 fibers were predominant and were much smaller than type 2 fibers on NADH stain. (I) Areas with mitochondrial loss and disrupted myofibrillar arrangement (arrows) were found on electron microscopy, which corresponded to core regions. (J) Materials with electron density similar to the Z-disk correlated with rod structures. Scale bar = 50 μm in A–G; 100 μm in H; 1 μm in I–J.
central core disease. Two previous reports describing muscle pathology in \textit{NEB}-related core-rod myopathy described well-demarcated cores and clustered rods in subsarcolemmal regions. On the other hand, \textit{KBTBD13}- and \textit{TPM2}-related disease show a different pathology, that is, nemaline rods are scattered through the sarcoplasm, and cores or minicores are randomly located with lack of clear demarcation. We find this difference remarkable, because in our patients, the muscle pathology resembled those of \textit{KBTBD13}- and \textit{TPM2}-related disease rather than previously reported cases with \textit{NEB} mutations. Patient 1 also showed additional pathologic abnormality, that is, severely disorganized intermyofibrillar networks with a moth-eaten appearance and the presence of ragged red fibers containing mitochondrial crystalline inclusions. However, the significance of these observations is unclear. Thus, our series demonstrates that the pathological features of \textit{NEB}-related core-rod myopathy are quite heterogeneous.

The muscle images of various congenital myopathies show specific patterns of muscle involvement and often provide important clues regarding the identities of causative genes. To date, congenital myopathies caused by \textit{NEB} mutations, such as nemaline myopathy and distal nebulin myopathy, usually show early involvement of anterior tibial muscles with relative preservation of gastrocnemius muscle until later stages. Previously described patients with \textit{NEB}-related core-rod myopathy have exhibited similar patterns, that is, prominent involvement of the anterior compartment of distal legs and axial muscles with selective preservation of gastrocnemius muscle. However, in both of our patients, medial gastrocnemius and soleus were most severely affected, while anterior tibial muscles were partially involved in patient 1 but completely spared in patient 2. Furthermore, predominant and early involvement of gastrocnemius seems to be unusual for \textit{NEB}-related myopathies.

On the other hand, asymmetrical muscle atrophy observed in patient 1 and his sibling is not uncommon in \textit{NEB}-related myopathies. The very mild phenotype observed in our patients was also surprising, because previous cases of \textit{NEB}-related core-rod myopathy have invariably had a severe phenotype. One patient presented with respiratory failure at birth (intermediate course), and another developed early respiratory insufficiency requiring ventilator support (typical congenital type). In another report of 6 patients with \textit{NEB}-related core-rod myopathy, 2 were severe and 3 were typical congenital types. Although our patient 1 experienced delayed motor milestones in infancy, both patients maintained independent ambulation without definite progression during follow-up, and neither developed respiratory distress. Core-rod myopathies associated with \textit{RYR1}, \textit{KBTBD13}, or \textit{TPM2} are dominantly inherited and generally cause mild disease with normal respiratory function and prolonged independent ambulation, although a lethal case has been described.

The correlation between residual nebulin amounts and clinical severity in \textit{NEB}-related myopathies has been discussed in several reports. In a case of nemaline myopathy, compound heterozygosity for a frameshift mutation and a splice site mutation (intron 13) caused a marked reduction in nebulin amount and resulted in a more severe phenotype than homozygous exon 55 deletions. The above-mentioned findings suggest clinical severity might be influenced by residual nebulin, which is probably determined by mutation type. Both of our patients were compound heterozygous for a frameshift mutation and a splice site mutation. Two frameshift mutations in each allele are expected to produce truncated proteins that would subsequently be degraded. Accordingly, the majority of residual protein detected by western blot was probably due to another allele carrying p.Ser8193Ser, because splice site mutations are often leaky and may
produce normal transcripts. Thus, the milder phenotypes shown in our patients could be explained by greater amounts of residual nebulin.

However, the presence a splice site mutation has not always correlated with higher nebulin amount and milder phenotype as shown in some other cases.\textsuperscript{14,20} In addition, the patients with a homozygous exon 55 deletion frequently show typical congenital or intermediate disease despite higher nebulin levels.\textsuperscript{21} Considering that patient 1 with a nebulin level similar to that of patients with homozygous exon 55 deletions\textsuperscript{17} had a much milder phenotype, we hypothesize that clinical severity is not likely to be only affected by residual nebulin amount, and that it is also determined by other unknown factors.

Mutation searches for congenital myopathies are a challenging task except for a few subgroups which are associated with a few small-sized causative genes or have mutational hot spots. In this regard, recent development of NGS technology is expected to make a significant contribution to the research in this field. NGS has already had a strong impact on identifying new causative genes in many familial cases and has nearly replaced traditional linkage analysis. However, its use is still limited because of too many variants identified and difficulties in proving pathogenicity of individual variants. And thus, it is still advisable to use NGS with traditional Sanger sequencing of target genes in every case. In core-rod myopathy, diverse clinical features and marked genetic heterogeneity made it difficult to target a specific causative gene, and use of whole exome sequencing in combination with Sanger sequencing of a few target genes brought us successful results.

In conclusion, we describe 2 patients with \textit{NEB}-related core-rod myopathy, who have a very mild phenotype and unusual pathologic features of rods and cores. This study shows that core-rod myopathy is a genetically heterogeneous entity with variable clinical and pathological findings, even among those with \textit{NEB} mutations. Our findings indicate that the residual nebulin amount is partly related to clinical phenotype severity. Further studies are required to further our understanding of the pathomechanism affecting clinical and pathological manifestations in \textit{NEB} related core-rod myopathy.

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