Distal myopathy with upper limb predominance caused by filamin C haploinsufficiency

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Running title: Filamin C mutation causing distal myopathy
Abstract

Objective: In this study, we investigated the detailed clinical findings and underlying genetic defect in three presumably related Bulgarian families displaying dominantly transmitted adult onset distal myopathy with upper limb predominance.

Methods: We performed neurological, electrophysiological, radiological and histopathological analyses of thirteen patients and thirteen at-risk but asymptomatic individuals from three generations. Genome-wide parametric linkage analysis was followed by bidirectional sequencing of the filamin C (FLNC) gene. We characterized the identified nonsense mutation at cDNA and protein level.

Results: Based on clinical findings, no known myopathy subtype was implicated in our distal myopathy patients. Light microscopic analysis of affected muscle tissue showed no specific hallmarks, however the electron microscopy revealed changes compatible with myofibrillar myopathy. Linkage studies delineated a 9.76 Mb region on chromosome 7q22.1-q35 containing filamin C (FLNC), a gene previously associated with myofibrillar myopathy. Mutation analysis revealed a novel c.5160delC frameshift deletion in all patients of the three families. The mutation results in a premature stop codon (p.Phe1720LeufsX63) that triggers nonsense-mediated mRNA decay. FLNC transcript levels were reduced in muscle and lymphoblast cells from affected subjects and partial loss of FLNC in muscle tissue was confirmed by protein analysis.

Conclusions: The FLNC mutation that we identified is distinct in terms of the associated phenotype, muscle morphology and underlying molecular mechanism, thus extending the currently recognized clinical and genetic spectrum of filaminopathies. We conclude that filamin C is a dosage-sensitive gene and that FLNC haploinsufficiency can cause a specific type of myopathy in humans.
Introduction

Distal myopathies are a diverse group of genetic disorders, various forms of which belong to the larger group of myofibrillar myopathies (MFM), dominantly inherited conditions usually beginning in adulthood and often having a limb-girdle weakness pattern. Pathognomonic microscopic findings in MFMs include aggregates containing myofibrillar degradation products and Z-disc related proteins. Distal MFMs are associated with mutations in DES, MYOT, ZASP and CRYAB.

Until recently, three FLNC mutations (p.Trp2710X, p.Val930_Thr933del and p.Lys899_Val904delinsValCys) were known to cause adult-onset MFM with a variable but commonly limb-girdle pattern of muscle weakness. Filamin C is a muscle-specific actin cross-linking protein involved in cytoskeleton organization and signalling. Functional and structural modeling suggests that mutant FLNC recruits FLNC-associated proteins, forming aggregates that gradually destabilize muscle tissue homeostasis.

Recently, two novel missense FLNC mutations (p.Met251Thr, p.Ala193Thr) were associated with a peculiar distal myopathy phenotype with hand muscle involvement and lack of pathological protein aggregation. These N-terminally located mutations were found to increase actin-binding affinity of FLNC. We report on three distantly related families with dominantly inherited distal myopathy with upper limb predominance caused by a novel loss-of-function FLNC mutation.
Filamin C mutation causing distal myopathy

Methods

Patients and evaluation

Thirteen patients (six men, seven women) from three pedigrees (2701, 2702, 2703) originating from the same village underwent detailed neurological examination (performed by VG, TC, VM, IT). Thirteen at-risk, asymptomatic relatives aged 17-87 years were also evaluated.

Standard nerve conduction studies (NCS) and concentric needle EMG were performed (by VG) using a Dantec–Keypoint portable electromyograph (Natus, Copenhagen, Denmark). Routine EKG and trans-thoracic cardiac ultrasound were performed (by MG).

Standard Protocol Approvals, Registrations, and Patient Consents

Written informed consent was obtained from all participants. This study was approved by institutional review boards of the Medical University-Sofia and the University of Antwerp.

MRI imaging

Patient 2701:IV.3 underwent MRI of lower and upper limbs and trunk muscles with a 1.5 Tesla MRI unit with a four-channel phased array body coil (Siemens Essenza, Erlangen, Germany). Images obtained were T1-weighted (T1w/SE, TR=545, TE=9.7), T2-weighted non-fat-saturated Spin Echo (T2w/FSE, TR=5202/3700, TE=96) and Turbo Inversion Recovery Magnitude with fat-saturation (T2 TIRM, TR=4020, TE=57/82, TI=170). Cross-sections from pelvic girdle, thighs and lower leg muscles were made with 7.5 mm slice thickness.
**Histological analysis and electron microscopy**

Open muscle biopsies were obtained from the medial head of the right gastrocnemius muscle in patients 2701:IV.3 and 2701:IV.4, and of the tibialis anterior muscle in patient 2702:IV.1. The latter sample was analyzed using standard histological and immunohistochemical light microscopy (LM) techniques. Antibodies used were against dystrophin (Dys1/2/3), dystroglycans (α/β), sarcoglycans (α/β/γ/δ), dysferlin (Ham1/Ham2), caveolin, spectrin, utrophin, merosin (80 kDa/200 kDa), 4H8-2-laminin α-2 chain, emerin, lamin A/C, laminin, vimentin, collagen VI, collagen IV, α-actinin, actin, desmin, myosin, MHC-developmental, titin, tropomyosin, telethonin and vinculin (details available upon request). Further stainings were performed using αB-crystallin (1:2000, NovoCastra, Wetzlar, Germany), myotilin (1:1000, NovoCastra) and FLNC (RR9019, 1:1000). The peroxidase-antiperoxidase technique was used for polyclonal antibodies and the avidin-biotin complex technique for monoclonal antibodies.

The biopsy samples of patients 2701:IV.3 and 2702:IV.1 were investigated using standard electron microscopy (EM). Semi-thin resin sections were stained with toluidine blue. Ultrathin sections were examined using a FEI CM10 transmission electron microscope (Philips, Amsterdam, The Netherlands) at 60 kV.

The biopsy from patient 2701:IV.4 was suitable only for western blotting.

**Linkage studies**

Genome-wide linkage analysis was performed using an in-house developed panel of 360 STR markers. Additional polymorphic markers were selected for fine-mapping (http://www.ncbi.nlm.nih.gov/unists/). Linkage was calculated using MLINK20 under an autosomal dominant model, equal male/female recombination rates and a 0.0001
Filamin C mutation causing distal myopathy

disease frequency. Asymptomatic individuals were classified as unaffected. Due to late disease onset, six penetrance classes were assigned: 0-20 years/0.6%, 21-35 years/9%, 36-45 years/37%, 46-55 years/74%, 56-65 years/94% and 66-80 years/99%.

Mutation analysis

Genomic DNA was extracted from peripheral blood using standard procedures. All 48 exons, exon-intron boundaries and 5’UTR of FLNC were amplified using primers designed with Primer3 v0.4.021 (table e-1, PCR conditions available upon request). PCR products were purified with ExoSAP-IT® (USB, Cleveland, USA) and bidirectionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Fragments were electrophoretically separated on an ABI3730xl DNA Analyzer (Applied Biosystems) and analyzed with SeqMan™ II (DNASTAR Inc., Madison, USA). Nucleotide and amino acid numbering followed mRNA (NM_001458.3) and protein (Q14315) FLNC sequences (www.ncbi.nlm.nih.gov). Mutation description followed HGVS nomenclature (http://www.hgvs.org/mutnomen).

RNA isolation and RT-PCR

Deep-frozen muscle tissue was crushed in a douncer, followed by phenol-chloroform RNA extraction. Lymphoblasts were homogenized by passage through a 21G syringe. Total RNA was purified using the RNeasy Mini Kit (Qiagen, Germantown, USA) and treated with DNase (TURBO DNA-free™ kit, Applied Biosystems). FLNC cDNA was synthesized by RT-PCR using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, USA) with random hexamers. Quantitative RT-PCR
(qRT-PCR) with SYBR green incorporation was performed on cDNA extracted from muscle tissue or lymphoblasts. The average Ct value obtained with multiple FLNC primers (FLNC_cDNA_EX21-22/28-29/35-36/37-38) was normalized against HMBS (HMBS_cDNA_EX1-2). For fragment analysis of muscle and lymphoblast FLNC transcripts, FLNC_cDNA_ex30F-FAM/30R primers were used at 35 or 38 PCR cycles respectively (table e-1).

Cycloheximide treatment
To inhibit nonsense-mediated mRNA decay (NMD)\textsuperscript{22}, lymphoblasts were incubated with 150 µg/ml cycloheximide (CHX) at 37°C for 4h. As a negative control, cells were treated with an equal volume of DMSO.

Western blotting
Proteins were extracted from frozen muscle by homogenization in E1A lysis buffer\textsuperscript{23} supplemented with a Complete Protease Inhibitor Cocktail tablet (Roche, Basel, Switzerland). Supernatant protein concentration was determined with the BCA Protein Assay (Thermo Scientific, Rockford, USA). The FLNC-containing pellet fraction was suspended in E1A buffer containing 2% SDS and sonicated. Extracts were boiled in NuPAGE\textsuperscript{®} LDS buffer, size-separated in a NuPAGE\textsuperscript{®} 3-8% tris-acetate gel (Invitrogen) and transferred to a nitrocellulose membrane. Membranes were immunoblotted with anti-FLNC antibodies (RR90\textsuperscript{19} and Abnova Corporation, Taipei, Taiwan), washed and incubated with horseradish peroxidase-labelled secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, USA). Western blot results were visualized using Amersham\textsuperscript{TM} ECL Plus (GE Healthcare, Waukesha, USA); bands were quantified using ImageJ software.
Filamin C mutation causing distal myopathy

(http://rsbweb.nih.gov/ij). Rabbit polyclonal histone H3 antibody (#9715, Cell Signaling Technology, Beverly, USA) was used for normalization.

Results

Clinical phenotype

Detailed clinical findings for thirteen affected individuals with distal myopathy and five asymptomatic mutation carriers are described in Table 1. No clinical differences were observed between males and females. The disease typically started in adulthood (average 41±11 years, range 20-57 years). Presenting symptoms were invariably distal, most frequently affecting upper limbs (8/13 patients) with lower limb involvement upon disease progression (Figure 1A). Muscle weakness remained restricted to distal limbs in 11/13 patients. Distal extensors were typically more severely affected than flexor muscles. Atrophy was seen mostly in interosseus muscles (especially the first dorsal), thenar, and anterior tibial muscles. Asymmetry was noted in 4/13 patients. All but one patient remain ambulatory to date. Distal deep tendon reflexes were reduced or absent. Distal sensory abnormalities were observed in six patients. Five mutation carriers (2701:IV.16, 2701:IV.14, 2702:IV.3, 2701:V.1, 2701:V.2) between 32-59 years of age were clinically normal.

Cardiac studies were performed on seven patients (Table 1). Only patient 2702:IV.1 showed cardiomyopathy on cardiac ultrasound with mild systolic dysfunction of the left ventricle, 50% ejection fraction and mild global hypokinesis. There were no data for coronary heart disease.

Serum creatine kinase (CK) levels in six patients and two asymptomatic carriers ranged from normal to six-fold elevated.
NCS in eight symptomatic individuals revealed diminished compound muscle action potential (CMAP) amplitudes in median, peroneal and tibial nerve recordings. Motor nerve conduction velocity (NCV) remained mostly normal. Sensory nerve action potentials (SNAP) were normal except in patients 2702:III.1 and 2701:IV.10. EMG in six patients revealed a myogenic pattern in five patients, and mixed myogenic and neurogenic in the anterior tibial muscle of patient 2702:III.1. Spontaneous activity (positive sharp waves) was recorded from one anterior tibial muscle (2701:IV.3).

**MRI studies**

MRI study (Figure 1B) of patient 2701:IV.3 disclosed a marked increase in signal intensity in triceps surae muscles bilaterally in both T1 and T2/TSE, consistent with fat replacement and lack of hyperintensity on TIRM sequences, indicating absence of muscular edema. Muscle volume was generally preserved. Tibialis anterior muscle was involved bilaterally, more pronounced on the left, as well as the medial head of gastrocnemius and soleus muscles, with fat replacement increasing from knees to ankles. Minor changes were seen in the distal part of peroneus longus and in the periphery of extensor digitorum longus. The posterior tibial muscle was preserved. In the proximal aspect of the lower limbs, the hamstrings and the gluteus maximus were affected, with sparing of the quadriceps, semitendinosus, gracilis, and sartorius. The shoulder girdle and upper arm muscles appeared normal.

**Histological features**

Biopsy 2702:IV.1 showed variations in muscle fiber size on LM (Figure 2). Fiber splitting, pyknotic nuclear clumps, a single necrotic fiber, and one fiber with a rimmed vacuole were also present. Endomysial fibrosis and a small group of
lobulated fibers close to a tendon were observed. Centralized nuclei were seen in 3% of the muscle fibers. No abnormal deposits, vacuoles, or hyaline inclusions were observed in the sarcoplasm. Oxidative enzymes staining showed focal areas of decreased activity. Myofibrillar ATPase, after preincubation at different pHs, showed a type 1 fiber predominance. Immunohistochemistry showed normal sarcolemmal proteins. There were no deposits of desmin, α-actinin, actin, titin, or tropomyosin. In the sarcoplasm, limited amounts of irregular αB-crystallin granulo-filamentous material were seen and very few fine granular deposits of myotilin. Apart from a single fiber containing FLNC-immunoreactive rods, no other FLNC immunoreactive deposits were detected.

The αB-crystallin and FLNC deposits did not co-localize with any of the other immunostained proteins. The corresponding fibers looked normal on trichrome and haematoxylin and eosin staining.

Ring-like fibers were observed upon toluidine-blue staining of semi-thin resin sections, although similar changes could not be detected on NADH-TR staining. Toluidine-stained sections of a second biopsy (patient 2701:IV.3) showed slight fiber size variations, rare isolated atrophic muscle fibers, few internalized nuclei, and fiber-splitting in the absence of structural myofibrillar lesions (data not shown).

On the ultrastructural level, the 2702:IV.1 biopsy showed a wide spectrum of ultrastructural changes, including myofibrillar disorganization (Figure 2J and inset) with diverse Z-disc abnormalities, such as streaming, densified striped material, small rods (insets of Figures 2J and 2K), and dappled bodies. Both preserved and disorganized ring-like muscle fibers were present, along with lobulated-like fibers with myofibrillar disorganization and fine granular sarcoplasmic masses (arrows, Figure 2K).
There were neither deposits of electron dense granular material nor accumulation of intermediate filaments. No rimmed vacuoles and no sarcoplasmic or intranuclear tubulofilamentous inclusions nor cytoplasmic bodies or spheroids were present. The ultrastructural changes observed in patient 2701:IV.3 were milder and included rare focal myofibrillar disorganization and Z-disc abnormalities, with streaming and flag-like semi-dense extensions, in addition to rare fibrillogranular-like aggregates (data not shown).

**Linkage analysis**

Genome-wide scan and two-point linkage analysis using penetrance classes showed linkage to chromosome 7q21.1-q35 in family 2701 (table e-2). The common geographic origin and phenotype among the families suggested a common pathogenic mutation. Genotype analysis revealed a common haplotype shared by all patients in the three families, consistent with a founder effect (figure e-1). The pedigrees were therefore combined in the consecutive fine-mapping analysis, yielding a cumulative LOD-score of $Z_{\text{max}}=3.54$ at $\theta=0$ at D7S635. Key recombinants (2701:IV.4, 2702:IV.1 and 2703:III.2) delineated the disease haplotype to a 9.76 Mb region between markers D7S650 and D7S649. All clinically affected patients and five asymptomatic individuals (2701:IV.14, 2701:IV.16, 2702:IV.3, 2701:V.1, 2701:V.2) carried the disease haplotype.

**Mutation analysis**

The candidate region contained 113 genes, of which only *FLNC* is predominantly expressed in striated muscles and is already associated with hereditary myopathy. Mutation screening of *FLNC* revealed an unknown c.5160delC variation in exon 30 of
Filamin C mutation causing distal myopathy

all disease haplotype carriers (Figure 3, e-1) resulting in a frameshift and a premature stop codon (p.Phe1720LeufsX63). The variation was absent from 177 Caucasian and 87 Bulgarian controls.

**FLNC expression studies**

We studied the functional effect of the c.5160delC mutation at the transcriptional level in muscle tissue of patients 2701:IV.4 and 2702:IV.1. Quantitative RT-PCR revealed a decrease of ~50% in FLNC mRNA (normalized against *HMBS*) in the patients relative to two unrelated controls (figure e-2A). Direct sequencing and length-dependent separation showed that the FLNC cDNA of patients consisted exclusively of wild type transcript (Figure 3, e-2B).

Although FLNC is predominantly expressed in striated muscle, we demonstrated FLNC transcript in lymphoblasts, thus providing an additional tool for expression studies. The FLNC mRNA reduction was rescued by cycloheximide treatment of lymphoblast cultures from patients 2701:IV.3 and 2702:IV.1 (figure e-2C), with a selective increase in c.5160delC mutant RNA (figure e-2D). This suggests that the FLNC mRNA decrease in our patients is due to NMD of the c.5160delC transcript.

Total protein extracts from muscle biopsies of patients 2701:IV.3, 2701:IV.4, 2702:IV.1 and four age-matched controls were immunoblotted with antibodies directed against epitopes located in the first two Ig-like domains (RR90) and in the utmost C-terminal part (Abnova Corporation) of the FLNC protein. Consistent with the diminished RNA levels, densitometric quantification of the FLNC protein signal intensity revealed a reduction of ~50% in FLNC expression in patient muscle tissue, as compared to controls (figure e-2E-F). No shorter protein fragments were detected,
even when loading excessive protein amounts and exposing the membrane for longer times (figure e-2F).

**Discussion**

We report on three distantly related Bulgarian families with distal myopathy due to a novel truncating *FLNC* mutation.

Three known *FLNC* mutations\(^{11-14}\) cause adult onset limb-girdle muscle weakness mainly affecting lower limbs, with some foot dorsiflexor and upper limb weakness upon disease progression. CK levels varied from normal to tenfold elevated. Some patients had cardiac or respiratory involvement.\(^{14}\)

Recently, a distal myopathy phenotype was described in two families with *FLNC* missense mutations.\(^{17}\) Disease started in the third decade of life with thenar muscle weakness, followed by posterior calf muscle involvement and slow progression to proximal involvement. None of the affected individuals had respiratory insufficiency, CK levels were normal to twofold increased and two patients displayed cardiomyopathy.

The c.5160delC mutation reported in our study causes adult-onset distal myopathy often characterized by upper limb predominance. Thenar muscles are consistently involved but finger extensors and foot dorsiflexors are the most severely affected. Onset age is slightly later and progression is milder than reported earlier\(^{17}\), with proximal weakness occurring only rarely. None of the subjects displayed respiratory insufficiency. Cardiomyopathy was documented in one patient.

In our patients, NCS typically revealed diminished CMAP amplitudes corresponding to recordings from atrophied distal muscles and myogenic changes on needle EMG. Clinical sensory loss was noted in 6/13 patients. Three of these underwent NCS,
Filamin C mutation causing distal myopathy

revealing abnormal sensory parameters and mixed myogenic/neurogenic pattern on needle EMG in one (2702:III.1), possibly suggesting a small fiber neuropathy in the other two. Although we have no strong arguments for a concomitant peripheral neuropathy in our families, it was previously suspected in patients carrying the FLNC p.Trp2710X mutation.11

In patients with limb-girdle filaminopathy, muscle pathology revealed typical MFM hallmarks (e.g., myofibril disorganization and large protein aggregates).12-14 In contrast, the recently reported distal myopathy patients showed unspecific myopathological abnormalities and consistent lack of myofibrillar aggregation on LM.17 We studied two biopsies from a severely affected tibialis anterior muscle (2702:IV.1) and a clinically preserved, but radiologically affected gastrocnemius muscle (2701:IV.3). Analogous to the previous study17, LM analysis revealed mild myopathic changes and a lack of myofibrillar aggregation. Very rare αB-crystallin and FLNC positive inclusions were noted, but they did not resemble typical MFM morphology. EM analysis, however, demonstrated various ultrastructural changes, including myofibrillar disorganizations with diverse Z-disc abnormalities. Although based on a limited sample, our morphological data suggests subtle myofibrillar changes in this particular form of filaminopathy. These minor changes may be compatible with those observed in MFM, although results of routine LM and immunohistochemistry are certainly not typical of this diagnosis.

Functional studies of the p.Trp2710X FLNC mutation showed that, although mutant proteins are expressed, they interact abnormally and recruit other cytoskeletal and sarcomeric proteins in large aggregates that ultimately disturb sarcomeric structure.16 This MFM pathology is consistent with a gain-of-toxic-function disease mechanism.
The two known distal myopathy FLNC mutations are N-terminally located amino acid substitutions that increase the actin-binding activity of the mutant protein. However, the precise pathomechanism is still unclear.\textsuperscript{17}

Our results broaden the genetic spectrum of filaminopathies by demonstrating the first FLNC frameshift mutation. The truncating c.5160delC mutation triggers NMD, thus decreasing total FLNC mRNA to \textasciitilde50\% of normal in patient’s muscle tissue and lymphocytes and halving the FLNC protein level in muscle tissue. These results are compatible with a loss-of-function mechanism leading to FLNC haploinsufficiency. Because FLNC is highly required in muscles for cross-linking actin with Z-disc proteins and the sarcolemma, it may be dosage sensitive. Alternatively, stoichiometric changes may influence FLNC’s signaling functions.\textsuperscript{24,25}

Haploinsufficiency has never been reported in patients carrying a FLNC mutation. A recent study reports a loss-of-function mouse model using a partially deleted FLNC gene, making it relevant for our c.5160delC-associated phenotype.\textsuperscript{26} Although mice homozygous for the truncated allele are not viable, heterozygous mice display no obvious myopathy phenotype.\textsuperscript{26} Like humans, the mice might display a milder phenotype that emerges only at older age. However, detailed phenotyping of heterozygous animals is currently lacking.

Our combined findings suggest that the unusual clinical presentation and absence of classical MFM-morphology in our families is associated with a null allele causing FLNC haploinsufficiency. Further functional studies are required to define whether the distal myopathy phenotype in the presented patients and in two recently reported families\textsuperscript{17} is caused by two independent molecular pathways, or whether the three mutations have a common downstream effect on functionality of muscle cells despite different triggering events.
Our study enlarges the clinical spectrum of filaminopathies and extends the histopathological and genetic heterogeneity of hereditary distal myopathies. Our findings stress the importance of EM for detecting myofibrillar abnormalities in patients suspected of a distal or myofibrillar myopathy, particularly in the absence of typical MFM features on LM. FLNC haploinsufficiency may cause subtle myofibrillar changes that risk going undetected on LM. Further morphologic studies in additional families could provide more insight into this matter.

For genetic counseling in distal myopathy families it is essential to consider the late disease onset and possible reduced penetrance of \textit{FLNC} mutations.
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Figure legends

Figure 1. Clinical and radiological features of patients with distal myopathy.

A. Clinical features of patients
   b. Weakness of the second, third and fourth finger dorsal flexion and muscle atrophy of the first dorsal interosseus in the youngest, most severely affected patient 2702:IV.1.
   c. Lower leg muscle atrophy (including tibialis anterior) in the same patient.
   d. Slight muscle atrophy of the distal lower legs in the same patient.

B. MR muscle imaging in patient 2701:IV.3.

Upper lower limbs. On the level of the hamstrings the affected muscles are: semimembranosus, biceps femoris long head, as well as gluteus maximus.

Lower leg muscles. In the distal lower leg we observed moderate to severe fatty muscle infiltration in the medial head of gastrocnemius and in soleus more pronounced distally. Minor changes are seen in the distal part of peroneus longus and in the periphery of extensor digitorum longus. The posterior tibial muscle is preserved and the anterior tibial muscle is affected. The muscle alteration presents with high signal intensity on both T1- and T2-weighted images and fatty infiltrated tissue has been suppressed on TIRM sequence with fat-saturation.

Figure 2. Histopathological findings in tibialis anterior muscle biopsy in patient 2702:IV.1.

A. Trichrome staining: variation in size of the muscle fibers without abnormal sarcoplasmatic deposits or inclusions. In one fiber, an accumulation of mitochondria
suggestive for a ragged-red fiber was present beside a subsarcolemmal area devoid of myofibrils and filled with a fine granular material (arrow); a single necrotic fiber is present (arrowhead).

B. Haematoxylin and eosin staining of the same localization at higher magnification showing the abnormal fiber with accumulation of mitochondria. Centralized nuclei are present in a few muscle fibers, accounting for 3% of the whole biopsy.

C. ATPase staining after preincubation at pH 4.6: numerical prevalence of type 1 fibers, some fibers have scalloped contours.

D. NADH-TR staining showing focal areas of decreased activity; no ring-like fibers were observed.

E. NADH-TR staining showing a group of small, lobulated fibers.

F. Toluidine blue staining of semi-thin resin embedded section: fiber-size variation with several atrophic muscle fibers and endomysial fibrosis; ring-like muscle fibers (arrow).

G. Toluidine blue staining of semi-thin resin embedded section showing ring-like fibers at higher magnification (arrow).

H. Immunohistochemistry staining with anti-αB-crystallin antibody: small amounts of irregular αB-crystallin positive granulo-filamentous deposits in a few fibers (NovoCastra, 1:2000, peroxidase-antiperoxidase technique).

I. Immunohistochemistry staining with anti-FLNC antibody showing a single fiber with a small accumulation of immunoreactive rods, no other immunoreactive deposits neither at subsarcolemmal nor at sarcoplasmic levels were found (RR90, 1:1000, peroxidase-antiperoxidase technique).
J. Ultrastructural analysis of a muscle fiber with a centralized nucleus showing myofibrillar disorganization with bundled (arrows) thin filaments (inset). Magnification: x 14500; Inset: x 41000.

K. Ultrastructural analysis of a lobulated-like muscle fiber with subsarcolemmal granular sarcoplasmic masses in which no specific electron-dense granulo-filamentous deposits were observed. Arrows indicate fine granular sarcoplasmic masses. Note few small rods (inset). Magnification: x 7250; Inset: x 21000.

Scale-bar = 100µm (A, C), scale-bar = 50µm (B, D-I)

**Figure 3. Sequencing analysis of the FLNC c.5160delC mutation.**

Sequence alignment of *FLNC* amplicons containing position c.5160 (arrow) in gDNA and cDNA prepared from respectively lymphoblasts and muscle tissue of patients 2701:IV.4 and 2702:IV.1 and an unaffected control. The patients’ gDNA analysis shows a heterozygous allele with c.5160 cytosine deletion that is not detected on the cDNA level.
References


<table>
<thead>
<tr>
<th>Patient</th>
<th>AAO/ALE</th>
<th>SAO</th>
<th>Muscle weakness</th>
<th>UL dist</th>
<th>UL prox</th>
<th>LL dist</th>
<th>LL prox</th>
<th>Walking</th>
<th>Sensory: pain and touch/VS and JPS</th>
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<td>na</td>
<td>pes cavus, finger flexion contractures, arterial hypertension / extrasystolic arrhythmia, valvular regurgitation</td>
<td></td>
</tr>
<tr>
<td>2701:III.15</td>
<td>45y/79y</td>
<td>weakness fingers</td>
<td>++ R&gt;L (45y)</td>
<td>-</td>
<td>++ L&gt;R (70y)</td>
<td>-</td>
<td>steppage gate</td>
<td>↓ dist UL/nl</td>
<td>93</td>
<td>na</td>
<td>na</td>
<td>no / na</td>
<td></td>
</tr>
<tr>
<td>2701:IV.3</td>
<td>49y/50y</td>
<td>weakness fingers</td>
<td>++ (49y)</td>
<td>-</td>
<td>++ (49y)</td>
<td>-</td>
<td>nl</td>
<td>nl/nl</td>
<td>116</td>
<td>CMAP absent in UL, SNAP and NCVs nl</td>
<td>myogenic</td>
<td>no / nl</td>
<td></td>
</tr>
<tr>
<td>2701:IV.4</td>
<td>54y/59y</td>
<td>walking difficulties</td>
<td>++ R&gt;L (56y)</td>
<td>-</td>
<td>++ L&gt;R (54y)</td>
<td>-</td>
<td>steppage gate</td>
<td>nl/nl</td>
<td>395</td>
<td>CMAP↓ tibial; SNAPs nl</td>
<td>myogenic</td>
<td>no / nl</td>
<td></td>
</tr>
<tr>
<td>2701:IV.5</td>
<td>36y/65y</td>
<td>weakness/paresthesia fingers</td>
<td>++ (36y)</td>
<td>-</td>
<td>++ (60y)</td>
<td>-</td>
<td>nl</td>
<td>↓ dist UL and LL/↓ dist UL and LL</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>no / na</td>
<td></td>
</tr>
<tr>
<td>2701:IV.10</td>
<td>35y/58y</td>
<td>weakness fingers</td>
<td>++ (35y)</td>
<td>-</td>
<td>++ (40y)</td>
<td>-</td>
<td>steppage gate</td>
<td>nl/nl</td>
<td>202</td>
<td>CMAP↓ in peroneal, tibial and median; SNAP nl in UL; sural SNAP absent</td>
<td>myogenic</td>
<td>Arterial hypertension / LV hypertrophy on cardiac ultrasound</td>
<td></td>
</tr>
<tr>
<td>2701:IV.12</td>
<td>45y/57y</td>
<td>weakness fingers</td>
<td>+ (45y)</td>
<td>-</td>
<td>+ (50y)</td>
<td>-</td>
<td>nl</td>
<td>↓ dist UL and LL/ nl</td>
<td>na</td>
<td>CMAP↓ distal LL and median, median NCV↓; SNAPs nl</td>
<td>na</td>
<td>Arterial hypertension / LV hypertrophy on cardiac ultrasound</td>
<td></td>
</tr>
<tr>
<td>2701:IV.15</td>
<td>50y/51y</td>
<td>weakness fingers</td>
<td>+ (50y)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nl</td>
<td>nl/nl</td>
<td>212</td>
<td>na</td>
<td>na</td>
<td>no / na</td>
<td></td>
</tr>
<tr>
<td>2701:IV.14</td>
<td>&gt;53y</td>
<td>asymptomatic,</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nl</td>
<td>nl/nl</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>2701:V.1</td>
<td>&gt;35y</td>
<td>asymptomatic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nl</td>
<td>nl/nl</td>
<td>167</td>
<td>nl</td>
<td>nl</td>
<td>no / na</td>
<td></td>
</tr>
<tr>
<td>2701:V.2</td>
<td>&gt;32y</td>
<td>asymptomatic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nl</td>
<td>nl/nl</td>
<td>315</td>
<td>nl</td>
<td>nl</td>
<td>no / na</td>
<td></td>
</tr>
<tr>
<td>2702:III.1</td>
<td>46y/63y</td>
<td>weakness fingers</td>
<td>++ (46y)</td>
<td>-</td>
<td>++ (50y)</td>
<td>-</td>
<td>mainly Parkinsonian</td>
<td>↓ dist UL/nl</td>
<td>na</td>
<td>median CMAP absent; tibial CMAP ↓, NCV ↓; median and ulnar</td>
<td>mixed myogenic/neurogenic in anterior tibial</td>
<td>no</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1: Clinical findings in patients and asymptomatic individuals carrying the p.Phe1720LeufsX63 FLNC mutation.

<table>
<thead>
<tr>
<th></th>
<th>Age 1</th>
<th>Age 2</th>
<th>Muscle Weakness</th>
<th>Volunteers</th>
<th>SNAP SNAP absent</th>
<th>CMAP SNAP absent</th>
<th>CMAP SNAP absent</th>
<th>NCVs and SNAP normal</th>
<th>Arterial Hypertension</th>
<th>LV Hypertrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2702:IV.1</td>
<td>22y/38y</td>
<td>Atrophy first dorsal interosseus</td>
<td>+++/+++L (22y)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>quickly fatigued</td>
<td>nl/nl</td>
<td>1189, 608, 569</td>
<td>CMAP absent UL/LL; NCVs and SNAP normal</td>
</tr>
<tr>
<td>2702:IV.2</td>
<td>34y/34y</td>
<td>Cold intolerance</td>
<td>hands/feet</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>nl</td>
<td>↓ dist UL and LL/nl</td>
<td>na</td>
</tr>
<tr>
<td>2702:IV.3</td>
<td>43y</td>
<td>Asymptomatic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nl</td>
<td>nl</td>
<td>na</td>
</tr>
</tbody>
</table>

AAO, age at onset; ALE, age at last examination; SAO, symptoms at onset; UL, upper limb; LL, lower limb; VS, vibration sense; JPS, joint position sense; CK, serum creatine kinase in units per liter, normal value 180 U/l for females and 200 U/l for males; NCS, nerve conduction studies; EMG, electromyography; muscle weakness tested according to the Medical Research Council scale, age between brackets after indicates the age at which weakness for that particular region was first noted; +, MRC 4 or 5; ++, MRC 3 or 4; ++++, MRC 0, 1 or 2; y, year; L, left, R, right; nl, normal; na, not available; ↓, reduced; dist, distal; -, absent; LV, left ventricle.
Filamin C mutation causing distal myopathy