Expanding the histopathological spectrum of CFL2-related myopathies

Short running title: Congenital myopathy related to new CFL2 mutations

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ABSTRACT

Congenital myopathies (CMs) caused by mutation in cofilin-2 gene (CFL2) show phenotypic heterogeneity ranging from early onset and rapid progressive forms to milder myopathy. Muscle histology is also heterogeneous showing rods and/or myofibrillar changes. Here, we report on three new cases, from two unrelated families, of severe CM related to novel homozygous or compound heterozygous loss-of-function mutations in CFL2. Peculiar histopathological changes showed nemaline bodies and thin filaments accumulations together to myofibrillar changes, which were evocative of the muscle findings observed in Cfl2/− knockout mouse model.

Key words: CFL2, cofilin-2, congenital myopathy, nemaline myopathy, sarcoplasmic aggregates, myofibrillar myopathy, NGS.

INTRODUCTION

Nemaline myopathies (NM), constitute a family of rare congenital disorders of skeletal muscle thin filaments characterized by the presence of nemaline bodies (rods) on muscle biopsy.¹ So far, mutations in 12 different genes have been reported to cause autosomal dominant (AD) or autosomal recessive (AR) forms of NM ²⁻¹⁴. Cofilin-2 is a widely expressed member of the AC group of proteins that regulate actin-filament dynamics ¹⁵. It binds to and depolymerizes filamentous F-actin, and negatively controls polymerization of monomeric G-actin in a pH-dependent manner ¹⁶.
Biallelic mutations in CFL2 are a rare cause of an AR form of NM (MIM #610687). The few reported cases are clinical and morphological heterogeneous leading to early-onset and rapidly progressive phenotype to a milder condition characterized by limb girdle and axial muscle weakness. Muscle histology shows features of nemaline or myofibrillar myopathy (MFM), as well as coexistence of both of these histopathological changes. Complete loss of cofilin-2 function is generally associated to a severe phenotype however the precise mechanism through which cofilin-2 defect leads to different structural abnormalities remains elusive.

Here we report on the clinical and myopathological findings in three patients from two unrelated families harboring novel mutations in CFL2, who presented an early-onset, severe form of congenital myopathy with peculiar histopathological findings of nemaline bodies and thin filaments accumulation.

Detailed information about methods are reported in appendix S1 (supplementary file).

**Ethics statement**

The clinical and molecular genetic study was performed in accordance with the Declaration of Helsinki. Studies of muscle biopsies and molecular genetic analysis have been approved by our ethics committee with written informed consent obtained for each patient.

**RESULTS**

**Clinical history and muscle pathology**

Patient 1 is a 7 year-old boy, first child of healthy consanguineous parents, presenting severe generalized muscle weakness that required continuous respiratory and nutritional support since birth. Neurological examination performed at the age of 4 years documented facial weakness that spared only extraocular muscles, macroglossia, and severe and generalized muscle weakness. Muscles masses appeared diffusely hypertrophic and with hardened consistency. Brain RMI, ECG and echocardiography were normal.
Patient 2 is a 1 year-old female, third child of non-consanguineous healthy parents. At age 20 days, she was admitted in ER for apnea episode and suction deficit with probable dysphagia. Clinical evaluation documented severe generalized hypotonia. Brain ultrasound, ECG and echocardiography were normal. Cranial nerves were spared. Only small distal movements of upper limbs were possible. Lower limbs were plegic, abducted and extra-rotated.

Patient 3, the older affected brother of Pt2, had a similar clinical picture and died at the age of 3 months for respiratory failure.

Clinical features of all patients are summarized in Table 1.

Muscle biopsy was performed at the age of 4 years in Pt1, 2 months in Pt2 and 23 days in Pt3. In all patients rods were observed. In Pt1 and Pt2 myopathic changes were more pronounced and small areas of myofibrillar dissolution together to thin filaments accumulation and cytoplasmic bodies (Pt1) were documented. ATPase reactions showed a type 1 fiber predominance only in Pt3 and immunostaining for dystrophin, sarcoglicans and merosin was normal (data not shown). Detailed morphological features are showed in Fig. 1-I-III.

**Laboratory results**

Whole Exome Sequencing (WES) in Pt1 and targeted resequencing using a custom gene panel for muscular diseases in Pt2, allowed us to identify three novel mutations in exon 2 of CFL2: c.256G>C; p.(Asp86His), homozygous in Pt1, and c.235G>T; p.(Asp79Tyr) and c.281delC; p.(Ser94LeufsTer6) in compound heterozygosity in Pt2. Sanger sequencing confirmed the mutations identified in probands and in Pt3, as well as heterozygosity for the mutations in their parents (Supplementary Fig. 1).

Immunohistochemical and western blot, performed in Pt1, confirmed the cofilin-2 reduction in muscle (Fig. 2-I) and slightly increased of CFL2 mRNA level was documented (Supplementary Fig. 2) thus providing evidence for the disruptive impact of the p.(Asp86His) change.
The structural impact of the two missense substitutions was also supported by the homology model of the human coflin-2 that shows that these residues are engaged in intramolecular interactions important for the folding of coflin-2 in the region mediating the binding to actin (Fig. 2-III).

**DISCUSSION**

Congenital myopathies related to CFL2 mutations are rare congenital disorders with wide clinical and myopathological heterogeneity. CFL2 encodes for coflin-2 that, together with coflin-1 and ADF/destrin, promotes actin-filament turnover in cells \(^{19,20}\). ADF and coflin-1 regulate actin filament dynamics at filament ends \(^{21}\), while coflin-2 has a higher affinity for filamentous actin and a slightly reduced actin-filament depolymerization activity \(^{20,22}\).

Several morphological features like nemaline bodies, minicores, concentric laminated bodies and areas of F-actin accumulation have been reported in patients carrying mutations in CFL2. These mixed phenotypes, overlapping nemaline and myofibrillar myopathies, suggest that expression of mutated forms of coflin-2 is causal to sarcomeric and extrasarcomeric cytoskeletal pathologies \(^{17}\), and highlights the functional importance of coflin-2 for F-actin depolymerizing activity and for the structural integrity of skeletal muscle tissue.

A knock-out mouse model of CFL2 was recently generated by Gurniak and collaborators \(^{23}\) to more precisely explore the physiological function of coflin-2. In the Cfl2\(^{-/-}\) model, myofibers and sarcomeric structures were initially formed correctly even in the absence of coflin-2. However, within a few days after birth, sarcomeric structures progressively undergo derangement, start to disintegrate, and pathological protein aggregates appear, without evidence of nemaline bodies formation. Thus, the Cfl2\(^{-/-}\) mouse rapidly develops all the hallmarks of a protein aggregate myopathy, similarly to what has been described in patients with homozygosity for the p.(Val7Met) change \(^{17}\).
We describe three patients from two unrelated families, who presented an early and severe form of CM related to mutations in CFL2. Only three CFL2-related NM families have been reported so far, all carrying homozygous mutations in CFL2 associated with partial or complete cofilin-2 deficiency. Similarly to the present findings, also in the previously reported cases has been demonstrated that the missense mutations had a pathogenetic role leading to a misfolding and accelerated protein degradation. Consistently, nonsense-mediated decay has been reported for CFL2 truncating changes, strongly pointing to loss of CFL2 function as the underlying mechanism of disease.

Muscle biopsies showed in all patients of our series myopathic changes with atrophic and hypertrophic myofibers, internalization of myonuclei and multiple nemaline bodies, and accumulation of thin filaments were also documented mainly in Pt1 and Pt2. Moreover, fibrosis, as well as sarcomeric disruption, were more pronounced in the oldest patient with the homozygous CFL2 p.(Asp86His) confirming the observation of Ockeloen and collaborators that the pathology associated with CFL2 mutations may evolve from NM phenotype at younger ages, towards one more closely pattern resembling MFM with increasing age. These results mirror the pathologic evolution of Cfl2−/− mice which, despite are normal at birth, rapidly develop a severe myopathy with thin filaments aggregates.

Conclusion

We report three new severe cases of CM related to novel inactivating mutations in CFL2. Only for the cases with null mutations, our patients were more severely affected than previously reported families with CFL2 missense mutations. Morphological features, consistent of thin filaments accumulations and myofibrillar changes, are evocative of the histopathological findings observed in Cfl2−/− mouse model. Structural modeling analysis supports the pathogenicity of the three novel CFL2 mutations and indicates that mutated residues are involved in correct folding of cofilin-2, thus compromising its interaction with actin filaments. This observation may explain the myofibrillar...
network disintegration that we observed in our patients and confirms that the activity of cofilin-2, to promote actin filament assembly, might also be important for the postnatal maintenance of sarcomeric structures.

References


FIGURE LEGENDS

Figure 1:

I: Thricrome Gomori (A, for Pt1, B for Pt2, C and F for Pt3) shows myopathic changes with fiber size variability, internal nuclei, presence of numerous small atrophic fibers and increased fat and connective tissue in all patients. In Pt1 prominent endomysial fibrosis and few fibers with vacuolar changes and cytoplasmatic bodies are observed (A). In Pt1 and Pt2 in larger fibres areas of green cytoplasmatic material can be noted (A-B). Nemaline bodies can be observed in Pt1 (A, arrow) and are numerous and more evident in Pt3 (C and F).
Abnormal sarcomere myofibrillar network and numerous area with uneven oxidative reaction can be documented in Pt1 and Pt2 with NADH staining (D and E respectively) and correspond to the area of thin filaments accumulation showed in Fig.1-II.

II: Fluorescence analysis reveals in both patients the myofibrillar network disorganization documented by the irregular immunostaining for desmin. Numerous areas positive for phalloidin and myotilin are indicative of F-actin-filaments accumulations. In both patients a diffuse reduction of cofillin-2 is also shown and spots of protein aggregations are observed in some fibers. Bar 50 μm.

III: Skeletal muscle ultrastructure shows in Pt1 (A-D) a wide array of myofibrillar changes including large areas of sarcomeric dissolution with accumulations of thin filaments and rods, cytoplasmic bodies, and autophagic vacuoles filled with membranous debris. Accumulations of thin filaments, nemaline bodies and M- and Z-lines misalignment were also observed in Pt2 (E-F).
Figure 2:

I: Western blot analysis from muscle of Pt1 shows a reduction >50% of cofilin-2 compared to the median value of two unaffected, age-matched control (Ct) muscle specimens (A-B) indicating accelerated degradation of the mutated protein.

II: (A) Multiple alignment between cofilin-2 and the other actin depolymerizing factors expressed in mouse and human (CFL1 and DSTN) showing that aspartate residues (D79 and D86, red arrows) altered in our patients are evolutionarily conserved among AC proteins. (B) Schematic diagram of CFL2 gene with previously reported (black) and presently identified (red) CFL2 mutations. Mutations reported in a compound heterozygous state are underlined.

III: Homology model of the human cofilin-2 bound to actin filaments based on the structure of cofilin-2 from chicken (Protein Data Bank, PDB 1TVJ) and superimposed onto the structure of the paralogous cofilin-1 bound to actin filaments (PDB 3J0S). The protein monomers are shown in different colors (the cofilin molecules are indicated by rounded arrows). Both the residues implied in the p.(Asp79Tyr) and p.(Asp86His) mutations are expected to modify protein folding and to cause defective binding of cofilin-2 to actin filaments. The p.(Ser94LeufsTer6) mutation implies the alteration/loss of a large portion of cofilin-2 (residues 94-166) necessary for the interaction of the protein with actin.
Table 1: Clinical features of patients presented in this study in comparison to previously reported families with CFL2 mutations. Abbreviations: y, year; m, month; NA, not available.
<table>
<thead>
<tr>
<th>Reference</th>
<th>This study</th>
<th>Agrawal et al., 2007</th>
<th>Ockeloen et al., 2012</th>
<th>Ong et al., 2014</th>
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</thead>
<tbody>
<tr>
<td>Patient (age at examination)</td>
<td>pt 1 (4ys) pt 2 (1 y) pt 3 (1m) pt 1 (16ys) pt 2 (2ys) pt 1 (21ys) pt 2 (5ys) pt 1 (1 m)</td>
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<tr>
<td>Onset</td>
<td>congenital congenital congenital congenital delayed motor milestone</td>
<td>breech presentation at birth</td>
<td>breech presentation at birth</td>
<td>respiratory distress and apnea at 5 days requiring intubation</td>
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<td>Floppy infant</td>
<td>yes yes yes yes yes no no no yes</td>
<td></td>
<td></td>
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<tr>
<td>Respiratory distress at birth</td>
<td>yes (tracheostomy at 2 months) yes (tracheostomy at 2 months)</td>
<td>yes no no no no + yes</td>
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<tr>
<td>Nutritional support</td>
<td>yes (gastrostomy at 2 months) yes (gastrostomy at 3 months) NA no no no no yes</td>
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<tr>
<td>Motor development</td>
<td>none none none delayed delayed delayed walking normal until age 2 yrs and 7 months compromised</td>
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<td>Facial weakness</td>
<td>yes no no NA NA high-arched palate, low-pitched voice no NA</td>
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<td>Contractures</td>
<td>severe ankles, wrists and fingers initial at ankles NA NA NA knees no NA</td>
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<tr>
<td>Scoliosis</td>
<td>yes, thoracolumbar scoliosis no no NA NA yes, severe kyphoscoliosis increased lordosis NA</td>
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<td>Muscle mass</td>
<td>hypertrophy wasting wasting NA NA wasting normal NA</td>
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<td>Distribution of weakness</td>
<td>diffuse and symmetric diffuse and symmetric (no head control, absence of tendon reflexes) diffuse and symmetric NA NA axial and proximal neck flexors, axial muscles, hip adductor, and periscapular muscles NA</td>
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<td>Best motor achievement and outcome</td>
<td>none none none can walk short distances but uses a wheelchair outside walking cannot run wheelchair dependent since age 21 waddling gait 24-h continuous ventilation support. Died at 12 months</td>
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<tr>
<td>Peculiar features</td>
<td>macroglossia - - - - head drop - -</td>
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<td>Cardiac involvement</td>
<td>- - - NA NA - -</td>
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<tr>
<td>CK</td>
<td>1500 600 800 NA NA normal NA mild elevation</td>
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