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 [8, 17, 18]. Muscle histology shows features of nemaline or myofibrillar myopathy (MFM), as well as coexistence of both of these histopathological changes [17]. 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 cofilin-2 that shows that these residues are engaged in intramolecular interactions important for the folding of cofilin-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 cofilin-2 that, together with cofilin-1 and ADF/destrin, promotes actin-filament turnover in cells [19, 20]. ADF and cofilin-1 regulate actin filament dynamics at filament ends [21], while cofilin-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 cofilin-2 is causal to sarcomeric and extrasarcomeric cytoskeletal pathologies [17], and highlights the functional importance of cofilin-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 cofilin-2. In the $Cfl2^{-/-}$ model, myofibers and sarcomeric structures were initially formed correctly even in the absence of cofilin-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 [8, 17]. Consistently, nonsense-mediated decay has been reported for *CFL2* truncating changes [18], 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 [17] 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 [23].

Conclusion

We report three new severe cases of CM related to novel inactivating mutations in *CFL2*. Only for the cases with null mutations[18], our patients were more severely affected than previously reported families with *CFL2* missense mutations [8, 17]. 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.

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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 cytoplasmic 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 cofilin-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**).

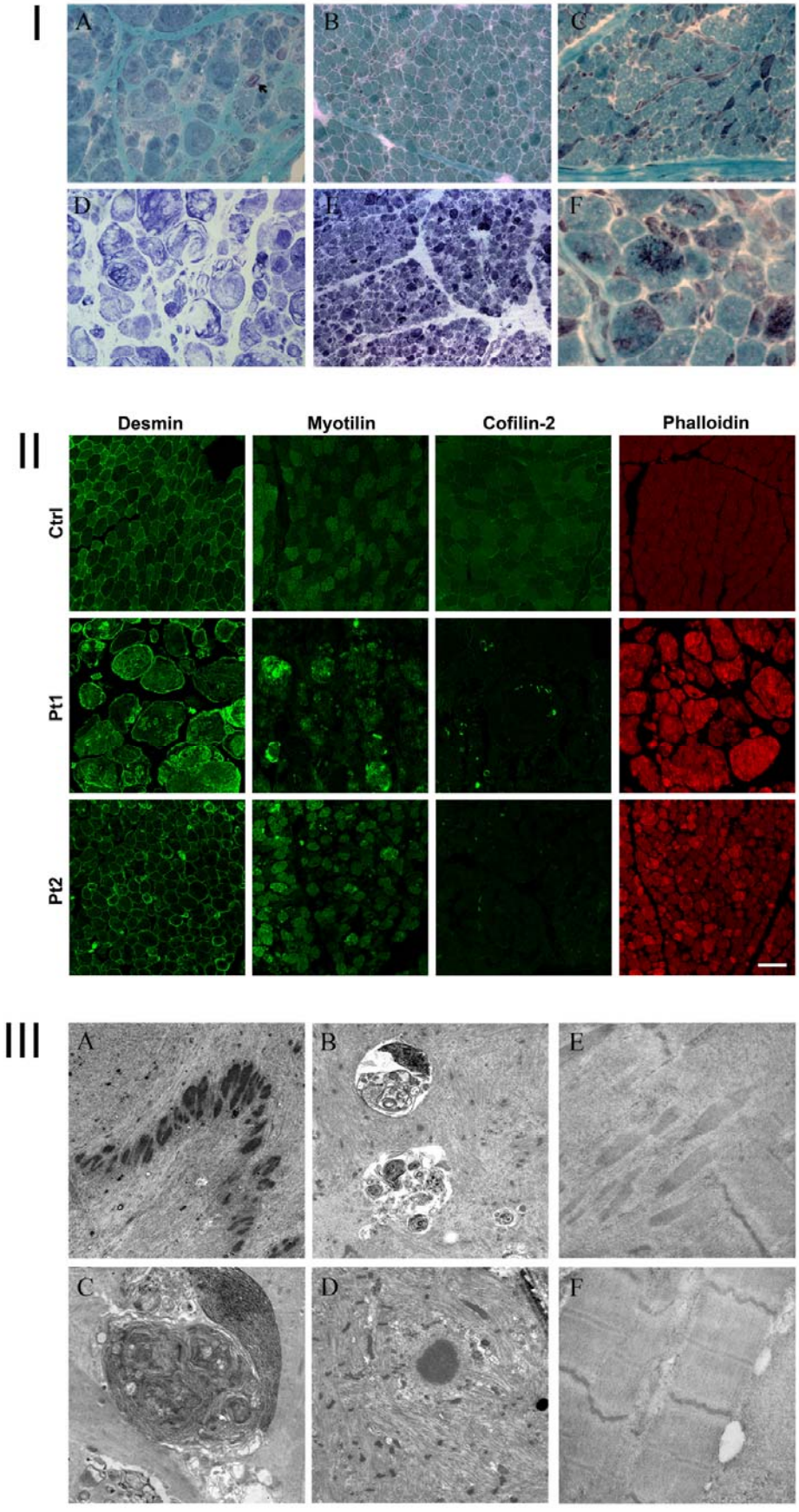

Figure 2:

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.

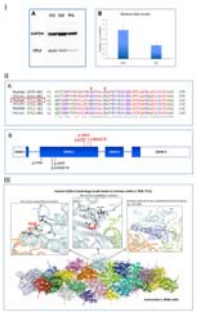

Table 1: Clinical features of patients presented in this study in comparison to previously reported families with *CFL2* mutations. Abbreviations: y, year; m, month; NA, not available.

Reference	**This study**			**Agrawal et al,2007**		**Ockeloen et al, 2012**		**Ong et al, 2014**
***CFL2* mutation**	**p.[(Asp86Asn)];[(Asp86Asn)]**	**p.[(Asp79Tyr)];[(Ser94LeufsX6)]**		**p.[(Ala35Thr)];[(Ala35Thr)]**		**p.[(Val7Met)];[(Val7Met)]**		**p.[(Lys34GlnfsX6)];[(Lys34GlnfsX6)]**
Patient (age at examination)	**pt 1 (4ys)**	**pt2 (1 y)**	**pt 3 (1m)**	**pt 1 (16ys)**	**pt2 (2ys)**	**pt 1 (21ys)**	**pt 2 (5ys)**	**pt 1 (1 m)**
Onset	congenital	congenital	congenital	congenital	delayed motor milestone	breech presentation at birth	breech presentation at birth	respiratory distress and apnea at 5 days requiring intubation
Floppy infant	yes	yes	yes	yes	no	no	no	yes
Respiratory distress at birth	yes (tracheostomy at 2 months)	yes (tracheostomy at 2 months)	yes	no	no	no	+	yes
Nutritional support	yes (gastrostomy at 2 months)	yes (gastrostomy at 3 months	NA	no	no	no	no	yes
Motor development	none	none	none	delayed	delayed	delayed walking	normal until age 2 yrs and 7 months	compromised
Facial weakness	yes	no	no	NA	NA	high-arched palate, low-pitched voice	no	NA
Contractures	severe ankles, wrists and fingers	initial at ankles	NA	NA	NA	knees	no	NA
Scoliosis	yes, thoracolumbar scoliosis	no	no	NA	NA	yes, severe kyphoscoliosis	increased lordosis	NA
Muscle mass	hypertrophy	wasting	wasting	NA	NA	wasting	normal	NA
Distribution of weakness	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 abductor, and periscapular muscles	NA
Best motor achievement and outcome	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
Peculiar features	macroglossia	-	-	-	-	head drop	-	-
Cardiac involvement	-	-	-	NA	NA	-	-	-
CK	1500	600	800	NA	NA	normal	NA	mild elevation