Short Report

Early childhood-onset axonal polyneuropathy, a new clinical AIFM1 form

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ABSTRACT

Objective To diagnose a couple of brothers with normal cognitive function who suffer from an early childhood-onset axonal polyneuropathy with exclusive involvement of motor fibers and to characterize major alterations in their fibroblasts.

Methods A customized gene panel was applied to identify the disease-causing mutation. The selected variants were further investigated by segregation analysis, pathogenicity prediction software and protein modeling. Expression analysis of the AIF protein, measurements of the number of apoptotic cells and observation of the cytoskeleton and mitochondrial network architecture were performed, respectively, by Western-blot, flow cytometry and immunofluorescence.

Results The AIFM1 c.629T>C (p.F210S) mutation was detected, which is predicted as damaging. Structural modeling showed that the mutation would affect the NAD binding and the dimerization domains of AIF. The mutation causes in the patients´ fibroblasts a decreased expression of the protein, altered morphology with aberrant cytoskeleton and a fragmented mitochondrial network, but without increased apoptosis.

Conclusions Increasingly new cases are described and the phenotypic spectrum of AIFM1-related disorders is expanding. The present case presents with an early childhood-onset axonal polyneuropathy, and therefore, the AIFM1 gene should be considered in the diagnosis of hereditary motor neuropathies.

Keywords Inherited peripheral neuropathies; AIFM1 gene; apoptosis inducing factor; gene panel; mitochondrial disorder.
INTRODUCTION

Inherited peripheral neuropathies (IPNs) are a group of disorders clinically and genetically highly heterogeneous. Mutations associated with IPNs (http://neuromuscular.wustl.edu/) in more than 80 genes have been already reported. Hereditary motor and sensory neuropathy (HMSN) or Charcot-Marie-Tooth (CMT) disease is closely related to distal hereditary motor neuropathy (dHMN) and, occasionally, some patients show additional disorder-related signs of amyotrophic lateral sclerosis (ALS) or spinal muscular atrophic (SMA). Therefore, in some cases the genetic, clinical and biochemical overlapping among IPN-associated genes can extremely complicate the differential diagnosis.

When the most common genes are discarded by conventional methods, targeted resequencing with a gene panel is being considered as a very powerful and cost-effective tool for diagnosis of inherited peripheral neuropathies.\(^1\)\(^2\) Using a gene panel that comprises 104 genes involved in CMT and related neuropathies, a novel \(AIFM1\) c.629C>T (p.F210S) mutation was identified in two brothers affected by an early childhood-onset polyneuropathy with exclusive motor involvement. The \(AIFM1\) gene encodes the apoptosis inducing factor (AIF), a mitochondrial FAD-dependent protein, and beyond its role in apoptosis, AIF takes part in mitochondrial metabolism.\(^3\)\(^-\)\(^5\)

\(AIFM1\) mutations are associated with a wide spectrum of clinical entities, including mitochondrial encephalomyopathy,\(^6\) prenatal ventriculomegaly;\(^7\) Cowchock syndrome;\(^8\) auditory neuropathy spectrum disorder;\(^9\) mitochondrial encephalomyopathy with ventriculomegaly;\(^10\) and spondyloepimetaphyseal dysplasia (SEMD) with mental retardation.\(^11\) This new case broadens the clinical spectrum of the \(AIFM1\)-related disorders.
METHODS

Patients’ history and electrophysiological data. Patients 1 (II:1) and 2 (II:2) are brothers, born to healthy unrelated parents of Bulgarian origin (see online supplementary figure S1). Their main complaint was progressive distal limb weakness that evolved over the years in a length-dependent manner. In both patients, electrophysiological studies revealed an axonal polyneuropathy with predominant involvement of motor fibers. Sensory amplitudes measurements were within the normal range (table 1).

Patient 1 is a 13 year-old boy. Motor milestone acquisition was normal during the first year of life, but at 16 months of age he was still unable to walk independently. Weakness in ankle dorsiflexion and areflexia were also noticed. At 5 years, thigh muscles were mildly affected, as well as both hands. When he was 6 years walking was achieved due to knee-ankle-foot orthoses, but he lost ambulation at 11 years. Patient 2 is an 8 year-old boy. At 18 months of age he had walking difficulties. He achieved independent ambulation beyond 2 years. By then he showed weakness and atrophy restricted to the distal muscles of lower limbs. Proximal muscles became affected later and both hands showed progressive weakness and wasting. He walks with ankle-foot orthoses and is still able to climb stairs while holding on to a rail.

Blood samples and skin biopsies were approved by ethics board of the Hospital 12 de Octubre (Madrid, Spain). The patient’s parents signed informed consent.

Genetic and in silico analysis. Patient 1 was investigated by gene panel Neuro104 (see online supplementary data) based on SureSelectQXT technology (Agilent Technologies, Santa Clara, CA, USA) for Illumina (San Diego, CA, USA). Sanger sequencing on an ABI Prism 3130XL analyzer (Applied Bioskysystems, Foster City, CA, USA) was performed for
validation and segregation analysis. To investigate the novelty of the variants, several databases were consulted (1000G, ESP6500, GnomAD, ClinVAR, and HGMD). Conservation of the residues was analyzed using Clustal Omega and the pathogenicity was predicted using SIFT, PolyPhen-2, and PROVEAN algorithms. The mutated protein was modeled by Coot software, using the structures 4BUR and 4BV6 (RCBS Protein Data Bank), which correspond to the human AIF in complex with FAD and with FAD and NAD, respectively.

**Cell culture, immunofluorescence and western blot.** Skin cultured fibroblasts derived from patients and their matched controls were grown in basal DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% (v/v) fetal bovine serum supplemented with 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

For immunofluorescence, cells were fixed in 4% paraformaldehyde and permeabilized in 0.25% Triton X-100. Subsequently, cells were blocked and immunostained in 5% horse-serum and finally, assembled with Vecta-Shield Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were visualized using a SP2- or SP8-Leica confocal microscope (Leica, Wetzlar, Germany). Anti-TOMM20 (Millipore, Bedford, MA, USA) was used at 1/250 dilution for mitochondria localization. The microfilament marker anti-actin (Sigma-Aldrich, St. Louis, MO, USA) was also used at 1/250 dilution to assess cell morphology changes.
Western blot analysis was performed by standard procedures, using fibroblasts’ lysates (25 μg protein per well), anti-AIF (at 1/500 dilution), and anti-actin (at 1/4000 dilution).

**Apoptosis measurements**

Fibroblasts were grown in basal DMEM medium during 3 or 6 days. Cells were detached from the culture flask using 0.05 trypsin-EDTA and then centrifuged at 300g for 5 min. After washing the cells with cold PBS, they were labelled for 15 min in the darkness with 5% Annexin V-FITC (BioLegend, San Diego, CA, USA) in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) and apoptosis measurements were done in a FC500 MPL Flow Cytometer (Beckman-Coulter).

**Measurement of an interconnectivity index of cellular mitochondrial networks**

A total of 150 cells per genotype were used to quantify the main area/perimeter ratio after growing for 48 hours in basal DMEM medium. MitoTracker® Red CMXRos (Thermo Fisher Scientific, Waltham, MA, USA) was used for labeling mitochondria.

**RESULTS**

After genetic testing using panel Neuro104 in patient 1, the only candidate change to be the disease-causing mutation was a novel variant in AIFM1, NM_004208:c.629T>C (NP_004199:p.F210S) in hemizygosis. Patient 2 and the healthy mother were carriers of the mutation, whereas the patients’ father does not harbor the variant. The F210 residue is highly conserved through evolution, and *in silico* prediction tools classified AIF^{F210S} as
damaging (not shown). The structural modeling of human AIF showed that F210 is localized in an alpha-loop on the protein surface (figure 1A), and its side chain interacts with P207 forming a stacking, and with Q206, which in turn forms another stacking with the side chains of F205 and F144 (figure 1B). The region between the peptide 205-210 and the side chain of F144 seems to generate a quite compacted structure. The mutation of a phenylalanine to serine at position 210 disrupts the hydrophobic interaction between F210 and P207 and, consequently, destabilizes the alpha-loop. Protein superposition of two different human AIF structures (figure 1A-B) suggests that a lack of constraints in this region could affect the interaction between 517–533 helix and the 190–202 β-hairpin regions, which is very important for the functional activity of the AIF protein. Thus, western blot analysis revealed decreased levels of AIF in the patients compared to controls (figure 1C).

We investigated the morphology of the patient’s fibroblasts, which is clearly aberrant by bright-field microscopy (see online supplementary figure S2). This alteration is confirmed by immunofluorescence, which has revealed a modified microfilament distribution in these cells (figure 1D). However, in the apoptotic assay, no significant differences are observed in the patients’ fibroblasts compared to the healthy control ones (figure 1E). On the other hand, using a specific antibody to detect the mitochondrial protein TOMM20, we have found fragmentation of the mitochondrial network (figure 1F). To confirm and to quantitatively assess it, we used the average area/perimeter ratio in which lower values indicate increased mitochondrial fragmentation. Thus, the mutant cells exhibit a statistically significant decrease of this rate (figure 1G), which supports the occurrence of mitochondrial fragmentation.
DISCUSSION

The variability of AIFM1 mutations leads to a broad range of complex clinical presentations.6-11 The patients have suffered from an early childhood-onset axonal polyneuropathy with exclusive involvement of motor fibers and have showed normal cognitive function, thus expanding the clinical spectrum of AIFM1 related-diseases. These data suggest that the AIFM1 gene should be considered in the diagnosis of hereditary motor neuropathies.

Several cell alterations have been described in patients with AIFM1 mutations. Thus, the p.E493V mutation increases caspase-independent apoptosis,8 while the p.R201del mutation leads to impaired OXPHOS.6 Although none of these two mutations alter AIF levels6,8, others (e.g. p.V243L, p.G262S or p.G338E) decrease it.10,13,14 For the new mutation here described, the genetic and in silico analysis indicate that the AIFM1 p.F210S mutation is likely deleterious. The analysis of the crystal structure of the mutated protein shows that p.F210S exerts a role in stabilizing the interaction between the regulatory peptide and the hairpin, which is relevant for the NAD binding and dimerization of AIF. AIFp.F210S expression is down-regulated and, although the mutation does not increase apoptosis, an aberrant cytoskeletal assembly and fragmented mitochondria are detected in patients’ fibroblasts, which could be related with a faulty mitochondrial respiration.15

In summary, these findings emphasize that AIF is involved in several and relevant cell functions and the distinct effects of different AIFM1 mutations could explain the variable clinical outcome. Thus, a strong decrease in the protein levels and/or in respiratory function would develop an early and severe mitochondrial disease, whereas weaker impairments would cause a slowly progressive neurological picture.13 Based on the results
shown here, our patients, who suffer from a severe early-onset motor neuropathy without cerebral involvement, could belong to the first group of AIFM1 disorders.
REFERENCES


Figure 1. Structural model of AIF<sup>F210S</sup> mutation and characterization of patients’ fibroblasts. (A) Structural model of two human AIF structures (4BUR and 4BVS), showing that F210 is localized in an alpha-loop on the protein surface. (B) Protein superposition of two different human AIF structures, suggesting that a lack of constraints could affect the proper dimerization. (C) Western-blot analysis of the Pt and two controls (Ct1: female; Ct2: male) total fibroblast lysates, which shows the decrease protein expression in the patient. (D) Immunofluorescence of microfilament using an actin-specific antibody showing an aberrant distribution in the proband’s fibroblasts. (E) Apoptotic assay in which no differences are observed between Pt and Ct, independently of the growing time. (F) Fibroblasts’ mitochondrial network, showing a fragmented pattern in the Pt, and a filamentous pattern in Ct. (G) Quantification of fibroblasts’ mitochondrial network, showing that the average area/perimeter ratio is significantly lower (P-value < 0.001) in the Pt compared to the Ct. In C-D, representative results of three independent experiments are shown. Pt: patient; Ct: healthy control.

Supplementary figure S1. Pedigree structure of the studied family. The arrow indicates the proband (Patient 1).

Supplementary figure S2. Bright-field microscopy visualization, showing normal healthy controls (Ct) and abnormal patient’s fibroblasts (Pt) morphology.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Onset</th>
<th>First clinical manifestations</th>
<th>AOW</th>
<th>Last clinical evaluation (March 2017)</th>
<th>Last electrophysiological evaluation (March 2017)</th>
<th>Current age: Functional status</th>
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<tbody>
<tr>
<td>II:1</td>
<td>16 months</td>
<td>Unable to walk independently. Weakness and distal muscles atrophy of lower limbs.</td>
<td>6 years (knee-ankle-foot orthoses is needed)</td>
<td>Severe distal weakness with prominent muscle atrophy. Proximal weakness is mild in upper limbs and moderate in lower limbs. Axial strength is preserved. Areflexia. Elbow contractures. Appreciation of position, vibration, light touch, temperature, and pinprick is normal.</td>
<td>NRR -Median (23.5 µV-58.5 m/s) -Radial (12.5 µV-47.4 m/s) -Sural (10 µV-NA) -Superficial peroneal (3.2 µV-36.8 m/s)</td>
<td>13 years: -Loss of ambulation: 11 years -Cognitive and cranial nerves functions are normal. -Formal visual and auditory assessment is normal.</td>
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<tr>
<td>II:2</td>
<td>18 months</td>
<td>Walking difficulties. Weakness and distal muscles atrophy of lower limbs.</td>
<td>2 years</td>
<td>Severe distal weakness and atrophy, and mild to moderate proximal weakness. Areflexia. Appreciation of position, vibration, light touch, temperature, and pinprick is normal.</td>
<td>NRR -Median (40.1 µV-56.4 m/s) -Cubital (37.3 µV – 50 m/s) -Sural (11.8 µV-67 m/s)</td>
<td>8 years: -He walks with a broad-based steppage gait and uses ankle-foot orthoses. -Cognitive and cranial nerves functions are normal. -Formal visual and auditory assessment is normal.</td>
</tr>
</tbody>
</table>

AOW, Age for onset of independent walking; MNCS, Motor nerve conduction study; SNCS, Sensory nerve conduction study; NRR, non-recordable response; NA, not available.
A

<table>
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<tr>
<th>Species</th>
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<th>Exon 6</th>
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<td>H. sapiens</td>
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<tr>
<td></td>
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<td>p.F210S</td>
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B

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<thead>
<tr>
<th>Protein</th>
<th>MutationTaster score (prediction)</th>
<th>PROVEAN score (prediction)</th>
<th>SIFT score (prediction)</th>
<th>Polyphen-2 score (prediction)</th>
<th>GERP score</th>
<th>PhyloP score</th>
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<tr>
<td>NP_004199:p.F210S</td>
<td>0.99 (disease causing)</td>
<td>-6.64 (deleterious)</td>
<td>0 (damaging)</td>
<td>0.531 (possibly damaging)</td>
<td>5.16</td>
<td>4.703</td>
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