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SHORT REPORTS

Spinocerebellar Ataxia Type 28—Phenotypic and Molecular Characterization of a Family with Heterozygous and Compound-Heterozygous Mutations in *AFG3L2*

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Abstract

While heterozygous mutations in the *AFG3L2* gene have been linked to spinocerebellar ataxia 28 (SCA28), homozygous mutations in the same gene can cause spastic ataxia 5 (SPAX5). *AFG3L2* encodes a mitochondrial ATP-dependent metalloprotease. We here report a SCA28 patient with biallelic *AFG3L2* variants and his heterozygous mother. The patient and his mother underwent a detailed neurological examination and fibroblast lines were established. The effect of the two missense variants on mitochondria was assessed by form factor analysis and quantification of mitochondrial proteins (TOMM70, complex V). The 39-year-old index patient presented with a slowly progressive cerebellar gait disorder for 19 years, bilateral ptosis, and dysarthria. A cranial MRI showed mild cerebellar atrophy. He carried two compound-heterozygous, rare, missense variants (c.1847A>G [p.Y616C], c.2167G>A [p.V723M]) in *AFG3L2*, while his mother was heterozygous for the first change that had previously been described in SPAX5. Altered mitochondrial morphology and interconnectivity, together with reduced protein levels of TOMM70 and complex V (ATPase), suggest mitochondrial structural defects in the patient's fibroblasts. No significant abnormalities were found in his mother's fibroblast cultures albeit all measurements were slightly below the control level. We here present a SCA28 patient with compound-heterozygous *AFG3L2* variants and demonstrate mitochondrial abnormalities in skin fibroblast cultures from this patient. Thus, *AFG3L2* variants should be considered in both slowly progressive ataxias and phenotypes with clinical features reminiscent of mitochondrial disease. Of note, ptosis was present in both mutation carriers and may serve as a red flag in the diagnosis of SCA28.

Keywords Spinocerebellar ataxia type 28 · AFG3L2 mutations · Ptosis

Introduction

Spinocerebellar ataxia type 28 (SCA28, OMIM #610246) is a rare neurodegenerative disorder characterized by a variable clinical phenotype with slowly progressive unsteady gait, mild cerebellar symptoms, and a variable age of onset from early childhood to the sixth decade [1]. It is inherited in an autosomal-dominant manner and caused by mutations in the *AFG3L2* gene (OMIM *604581) [2]. So far, about 30 different *AFG3L2* variants were reported in ~30 SCA28 families [3–5]. Of note, homozygous mutations of *AFG3L2* have been found in autosomal recessive spastic ataxia 5 (SPAX5) [6–8], characterized by early-onset spasticity resulting in significantly impaired ambulation, cerebellar ataxia, oculomotor apraxia, dystonia, and myoclonic epilepsy (OMIM #614487). *AFG3L2* encodes a mitochondrial ATP-dependent metalloprotease (m-AAA protease), which is highly expressed in Purkinje cells and plays a role in protein degradation and regulation of ribosome assembly [4]. The m-AAA protease is composed of either an *AFG3L2* homo-oligomeric isoenzyme or a hetero-oligomeric complex with paraplegin, a protein that

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is mutated in another autosomal recessive spastic paraplegia, i.e., type 7 (SPG7, OMIM #607259). Pleiotropic cellular phenotypes have been observed upon loss of the m-AAA protease function including fragmentation of the mitochondrial network, reduced assembly of respiratory complexes, disturbance of mitochondrial transport, dysregulation of mitochondrial Ca^{2+} influx, and Ca^{2+}-induced cell death [3, 6, 9, 10]. It has been shown that neurons are highly susceptible to decreased m-AAA protease levels, and cannot survive AFG3L2 deficiency [11]. Additionally, mitochondrial swelling and neuronal loss have been demonstrated in SCA28 patients and *afg3l2*-knockout mice [4, 9].

Here, we report a 39-year-old cerebellar ataxia patient harboring biallelic variants in *AFG3L2*. Mitochondrial properties were investigated in patient-derived cultured fibroblasts to determine damaging effects of the two variants.

Patients and Methods

Patient

The index patient and his mother were recruited at the Department of Neurology at the University Hospital Schleswig-Holstein, Campus Lübeck. After written informed consent was obtained, they underwent a detailed neurological examination. Blood samples and fibroblasts were collected. The ethics committee of the University of Lübeck approved the study.

Functional Assessment of AFG3L2 in Patient-Derived Fibroblast Lines

Fibroblast cultures were established from the patient (L-10182) and his mother (L-11388) from skin biopsies. In addition, a fibroblast line from an unrelated healthy control (L-2132) was included.

The "form factor" as a measure of the integrity of the mitochondrial network was determined as described [12]. In brief, the mitochondrial network in fibroblasts was stained with an anti-GRP75 antibody (1:1000, Abcam, Cambridge, MA) in combination with the Zenon immunolabeling kit (Invitrogen, Carlsbad, CA). Based on single-cell images, mitochondrial area and outline were measured and the form factor was calculated (defined as $[P_m^2]/[4\pi A_m]$), with P_m being the length of the mitochondrial outline and A_m being the area of the mitochondrion. For each individual, ten images from two independent experiments were analyzed.

Western blot analyses were performed with extracted proteins from cell pellets using SDS extraction buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% DOC, 1% NP-40, and 0.1% SDS). Gels were blotted onto nitrocellulose membranes. Antibodies used for western blotting were anti-GRP75 antibody (1:1000, Abcam, Cambridge, MA), anti-complex V (1:1000, Abcam, Cambridge, MA; raised against purified mitochondrial Complex V (Cow)), anti-β-actin (1:500000, Sigma-Aldrich), and anti-TOMM70 (1:1000, Santa Cruz Biotechnology). Differences were statistically analyzed using analysis of variance (ANOVA) with Bonferroni post hoc test.

TOPO Cloning

As the father of the patient was not available for genetic testing, subcloning and sequencing of the patient's cDNA verified the compound-heterozygous state of the two variants. RNA was extracted from cultured skin fibroblasts and cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA). *AFG3L2*-PCR fragments of 543 bp (F: 5′-GTATCTGGAGCACGCAGAC-3′; R: 5′-CCAGTGCCTTCCACAAATTCTTC-3′) were cloned into TOPO TA Cloning™ vectors (Thermo Fisher) and transformed into One Shot™ TOP10 electrocompetent *E. coli* (Thermo Fisher). Ten independent colonies were picked randomly and sequenced.

Results

Clinical Assessment

The 39-year-old male patient presented with a slowly progressive gait impairment. Medical history revealed congenital cleft lip and palate, bifid uvula, an over-extendibility of joints (e.g., thumb remarkably stretchable), gait impairment since the age of 20 years, and mild hyperthyroidism (diffuse struma), requiring no medical treatment at the time of presentation. Cognitive development was normal. After obtaining a high school degree, he finished a professional training as an IT specialist. The neurological examination revealed dysarthria, bilateral ptosis, slightly hypermetric saccades, mild arm dysmetria, and moderate gait ataxia (SARA score 11/40, Table 1, Video 1). He had no muscle weakness or atrophies, deep tendon reflexes were normal to brisk, and sensory examinations were unremarkable. Extensive laboratory tests, including for lactate, creatine kinase, vitamins A, B, E, alpha-fetoprotein, revealed normal results. There was no evidence for acanthocytes. Cerebrospinal fluid was normal in terms of cell count and glucose, protein, lactate, and immunoglobulin levels. EEG was also normal. A cranial MRI showed mild cerebellar atrophy. Ophthalmological examinations revealed a visual acuity of 0.8 for both eyes without retinal abnormalities. Upon abdominal sonography, a slight hepatosplenomegaly was found. There was no clinical information available on the Romanian father or his relatives. Maternal family history was unremarkable for neurological/neurodegenerative or psychiatric disorders.

Table 1 Clinical characteristics of our family in comparison with previously published patients with the p.Y616C mutation

	Our index patient	Our index patient's mother	Index patient of Pierson et al.	Index patient's mother of Pierson et al.
Genotype	c.2167G>A p.V723M c.1847A>G p.Y616C (comp. heterozygous)	c.1847A>G p.Y616C (heterozygous)	c.1847A>G p.Y616C (homozygous)	c.1847A>G p.Y616C (heterozygous)
Origin	German/Romanian	German	Hispanic	Hispanic
Age at exam (years)	39	58	Teenager	39
Age at onset (years)	20	n/a	2	n/a
SARA score	11/40	2/40	n/a	n/a
Limb ataxia	Yes	No	Yes	No
Gait ataxia	Yes	No	Yes	No
Dysarthria	Yes	No	Yes	No
Gaze-evoked nystagmus	No	No	Not indicated	No
Slowing of saccades	No	No	Not indicated	No
Oculomotor apraxia	No	No	Yes	No
Ophthalmoparesis	No	No	Yes	No
Ptosis	Yes	Yes	Yes	No
Dystonia	No	No	Yes	No
Stimulus-induced myoclonus	No	No	Yes	No
Reflexes	Reduced-normal in upper extremities; elevated in lower extremities	Normal	Not indicated	Normal
Muscle atrophy	No	No	Distal extremities	No
Spasticity	No	No	Yes	No
Paresis	No	No	Spastic paraparesis	No
Fundoscopic/ ophthalmological examination	Unremarkable	n/a	Unremarkable	Unremarkable
Nerve conduction studies	Bilateral conduction disturbance of peroneal nerves (as a result of compression syndrome)	n/a	Axonal sensorimotor peripheral neuropathy in bilateral lower extremities	Normal
Electromyography	Normal	n/a	Active denervation, polyphasic motor units	Normal
MRI	Mild cerebellar atrophy	No cerebellar atrophy	Moderate cerebellar atrophy	Mild cerebellar atrophy
EEG	Unremarkable alpha-EEG	n/a	Diffuse slowing/ disorganization, fronto-central spike/waves	n/a
Epilepsy	No	No	Progressive myoclonic epilepsy	No
Cognition (MoCA score)	Normal (30/30)	Normal (28/30)	Normal	Normal
Miscellaneous	Congenital cleft lip and palate, bifid uvula, over-extendibility of joints, hyperthyroidosis, hepatosplenomegaly	Abnormal posture due to chronic back pain, tendon rupture and synovitis, migraine	Parents are first cousins, affected younger brother had a more severe disease course and deceased at age of 13	n/a

The German mother was asymptomatic with respect to ataxia. At clinical examination at the age of 58 years, she had slight bilateral ptosis and difficulties in tandem walk and fast alternating hand movements (resulting in 2 points in the SARA score) mainly due to chronic pain (attributable to scoliosis and osteoblastic spinal bone changes) rather than due to

neurological abnormalities. She showed abnormal posture due to chronic back pain; however, neither dystonia nor abnormalities in terms of muscle tone were present. She further reported an asymptomatic aneurysm of the left mid cerebral artery, migraine headaches since the age of 52 years, and recurrent depressive episodes.

Genetic Testing

After exclusion of mutations linked to Friedreich ataxia; ataxia-telangiectasia; ataxia with oculomotor apraxia type 1 and 2; spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7, 8, 12, and 17; and Niemann-Pick disease type C (*NPC1* or *NPC2*); a total of 124 ataxia genes were analyzed using next-generation sequencing (gene panel, MiSeq 2 × 150 bp paired-end sequencing; Illumina). This revealed two heterozygous variants in the *AFG3L2* gene in the index patient, a previously reported variant in a SPAX5 patient [6] (c.1847A>G; p.Y616C) and a novel variant (c.2167G>A; p.V723M, Table 1). Both variants are extremely rare in public databases (https://gnomad.broadinstitute.org/gene/ENSG00000141385, minor allele frequency < 0.0002), conserved in higher vertebrates including mammals, and predicted to be pathogenic/damaging by several prediction tools such as PolyPhen ("probably damaging") [13], MutationTaster ("disease causing") [14], and a CADD score of 32 and 25.6, respectively [15]. His mother carried only the c.1847A>G change. Since the father of the patient was not available for testing, we confirmed the presence of the variants on different alleles (compound-heterozygous) by TOPO cloning (Fig. 1a). Whether the second variant was inherited from the father or arose de novo cannot be determined without a sample of the father. Likewise, the clinical status of the father is unknown.

Functional Analyses

To assess the effect of the two missense variants on mitochondrial properties, we first analyzed mitochondrial morphology and interconnectivity by form factor analysis. This revealed a significant reduction in the degree of mitochondrial branching

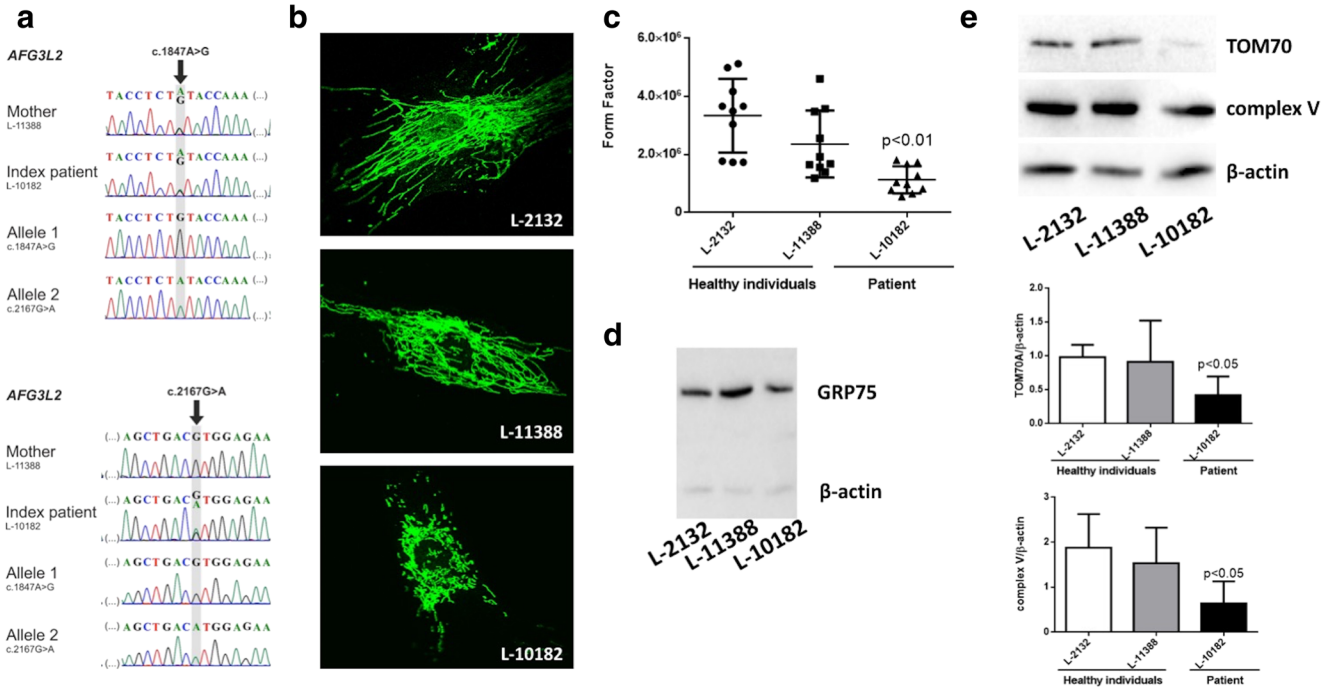

Fig. 1 Genetic findings and altered mitochondrial morphology and function in *AFG3L2* mutant fibroblasts. **a** Electropherogram of *AFG3L2* (NM_006796) with one heterozygous mutation (c.1847A>G) in the mother (L-11388) and two heterozygous mutations (c.1847A>G; c.2167G>A) in the patient (L-10182). The two alleles of the index patient were separated by subcloning into vectors. This demonstrated that both variants are located on different alleles and thus are present in a compound-heterozygous state. **b** Representative pictures of the mitochondrial network under basal conditions by confocal microscopy in fixed cells immunostained with anti-GRP75 (green) are shown for a control fibroblast line (L-2132), the patient (L-10182), and patient's mother line (L-11388). **c** The form factor as a measure for mitochondrial interconnectivity was calculated (10 cells each, $n = 2$). Each dot represents measurement in a single cell; and the mean, the minimum and the maximum value, and the interquartile range of the investigated individuals are shown ($p < 0.01$ refers to a high statistically significant difference between the patient (L-10182) and a control fibroblast line (L-2132)). **d** Western blot analysis shows mitochondrial GRP75 levels with ß-actin as loading control and respective quantification using ImageJ. **e** Western blot of TOMM70 and Complex V protein levels with β-actin as loading control. Quantification of the blot was done using ImageJ. Mean values and the standard deviations of three different experiments of the investigated individuals are shown ($p < 0.05$ refers to a statistically significant difference between the patient (L-10182) and a control fibroblast line (L-2132)).

in the patient ($p < 0.01$) suggesting an increased number of fragmented mitochondria in patient's fibroblasts (Fig. 1b, c). Further, the level of GRP75 was also reduced in the patient (Fig. 1d). These changes were accompanied by decreased levels of two mitochondrial proteins: TOMM70 (the importer receptor for translocases in the outer mitochondrial membrane complex) and respiratory chain complex V (ATPase) (Fig. 1e), confirming mitochondrial structural defects, and suggesting suppressed mitochondrial function. Importantly, no significant abnormalities were found in the mother's fibroblasts; however, all measurements were below the control level (Fig. 1b–e).

Discussion

This is, to our knowledge, the first report of a patient with slowly progressive ataxia and bilateral ptosis linked to biallelic variants in the *AFG3L2* gene. Previously reported SCA28 patients had a single heterozygous *AFG3L2* variant only. While the p.Y616C variant has been linked to SPAX5 in the homozygous state [10], its role in combination with another *AFG3L2* variant has not yet been explored. The heterozygous parents of the previously described siblings were reported to have normal neurological and ophthalmological examinations and an MRI scan of the father was also unremarkable. In contrast, the mother in that family showed mild cerebellar atrophy [10]. Although the activity of AFG3L2^{Y616C} was decreased, the expression of one wild-type allele appeared to provide a sufficient amount of m-AAA protease activity to remain below a theoretical threshold for cerebellar dysfunction in the healthy parents [10]. Similarly, we observed slight changes in mitochondrial morphology and network in the heterozygous, asymptomatic mother of our patient (Fig. 1) who did not have overt ataxia, although she had ptosis and obtained 2 points on the SARA score (Table 1). Whether there is a correlation of subtle clinical signs with slight changes in the functional assays in the mother cannot be determined based on a single case. On the other hand, our functional data further demonstrated that the p.V723M variant on the second allele is adding to significant mitochondrial structural defects and the SCA28 phenotype in the index patient. Of note, p.V723M is located in the proteolytic domain. Since the father was unavailable for clinical examination, we cannot comment further on the role of p.V723M in the heterozygous state.

Conclusion

We here present the first carrier of biallelic missense variants in *AFG3L2*, causing mitochondrial abnormalities and a SCA28 phenotype. *AFG3L2* mutations should be considered an important differential diagnosis in both slowly progressive ataxias and phenotypes reminiscent of mitochondrial disease. Of note, both mutation carriers in our study had ptosis which has previously been reported in SCA28 patient and thus could serve as a red flag in the diagnosis of SCA28 due to *AGF3L2* mutations.

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Compliance with Ethical Standards The study was approved by the Ethics Committee of the University of Lübeck, Germany (AZ 16-068).

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These authors declare that they have no conflict of interest:
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