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ARTICLE

Biallelic variants in AGTPBP1, involved in tubulin deglutamylation, are associated with cerebellar degeneration and motor neuropathy

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Abstract

The ATP/GTP-Binding Protein 1 (*AGTPBP1*) gene (OMIM *606830) catalyzes deglutamylation of polyglutamylated proteins, and its deficiency manifests by cerebellar ataxia and peripheral neuropathy in mice and lower motor neuron-like disease in sheep. In the mutant mice, cerebellar atrophy due to Purkinje cell degeneration is observed, likely due to increased tubulin polyglutamylation in affected brain areas. We report two unrelated individuals who presented with early onset cerebellar atrophy, developmental arrest with progressive muscle weakness, and feeding and respiratory difficulties, accompanied by severe motor neuronopathy. Whole exome sequencing followed by segregation analysis in the families and cDNA studies revealed deleterious biallelic variants in the *AGTPBP1* gene. We conclude that complete loss-of-function of AGTPBP1 in humans, just like in mice and sheep, is associated with cerebellar and motor neuron disease, reminiscent of Pontocerebellar Hypoplasia Type 1 (PCH1).

Introduction

Pontocerebellar hypoplasia (PCH) is a group of rare, inherited progressive neurodegenerative disorders [1]. To date, 11 different subtypes have been defined by clinical, neuroradiological and genetic features. Common characteristics include hypoplasia/atrophy of the cerebellum and pons, progressive microcephaly, variable cerebral involvement and autosomal recessive inheritance. Affected individuals suffer from severe cognitive and motor deficits. Seventeen causative genes have been reported, most of which function in RNA processing or translation. The first patient with pontocerebellar hypoplasia (PCH) and spinal motor neurodegeneration as seen in Werdnig-Hoffman disease (or spinal muscular atrophy, SMA) was reported in 1961 by Norman [2], followed by additional reports of similar patients [3, 4]. These reports enabled delineation of a distinct subtype of PCH characterized by pontocerebellar hypoplasia or atrophy with bulbar and spinal motor neurodegeneration, to be classified as PCH type 1. Heretofore, variants in five genes have been associated with PCH type 1, with ~50% of patients carrying variants in *EXOSC3* [5–7]. *EXOSC3* encodes for component 3 of the exosome complex, which is involved in RNA processing, and variants are associated with variable degrees of cerebellar hypoplasia and pons involvement.

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Variants in genes encoding other components of the exosome, *EXOSC8* and *EXOSC9*, are similarly associated with psychomotor retardation, spasticity, hearing and vision impairment and severe muscle weakness due to motor neuron degeneration [8, 9]. Brain imaging shows progressive cerebellar atrophy with relative sparing of the brainstem. Variants in genes encoding non-exosomal components were also associated with PCH and SMA-like disease, as in the vaccinia-related kinase 1 (*VRK1*) [10–12] and the *SLC25A46* genes [13, 14]. VRK1 was previously reported to be of importance for the maintenance of amyloid-β precursor protein [15], whereas *SLC25A46* encodes a mitochondrial outer membrane carrier which is involved in mitochondrial fission and fusion and maintenance of the mitochondrial cristae [16]. Thus PCH1 is a common phenotype to perturbations in several essential, but seemingly unrelated, mechanisms.

Here we report on the results of molecular investigation of two affected individuals from two unrelated families, who suffered from cerebellar hypoplasia and SMA-like disease. Our data adds a new mechanism to this group of disorders.

Materials and methods

Exome analysis

Following informed consent, exome analysis was pursued on DNA extracted from whole blood. Exome analysis of patient 1 and his parents and of patient 2 was performed on exon targets captured using SureSelect Human All Exon 50 Mb V5 Kit (Agilent Technologies). Sequences were determined by HiSeq2500 (Illumina, San Diego, California, USA) as 100–125-bp paired-end runs, with a mean coverage of 75×. The full sequencing methodology and variant interpretation protocol were previously described [17].

Segregation analysis

Amplicons containing the *AGTPBP1* variants were amplified by conventional PCR, and analyzed by Sanger dideoxy nucleotide sequencing. The following primers were used for **Family 1**: forward primer: 5′- TGCTCATCTGTTTAAA TGCAATG-3′ and reverse primer: 5′-TTTTCCACAGTT AAGATGCCTG-3′. For **Family 2**, *AGTPBP1* exon 16 (NM_001286715.1) was amplified with forward primer: 5′-TTGAGTCTGGGAATCTGCG-3′ and reverse primer: 5′-TTTTCCACAGTTAAGATGCCTG-3′. *AGTPBP1* exon 20 was amplified with forward primer: 5′-GACATTTCT AAAGCCCCAAAG-3′ and reverse primer: 5′-AACCTG GACATTGTTATTTCAAC-3′. Exons were numbered sequentially according to their delineation in NM_001286715.1, and as per UCSC genome browser (URL: genome.ucsc.edu) for this RefSeq transcript.

RNA studies

RNA was isolated from fresh lymphocytes of the patients, parents and control using Quick-RNA™ Whole Blood kit (Zymo Research) and cDNA was prepared using Maxima™ 1st strand cDNA synthesis kit (Thermo Scientific). For Family 1, the region encompassing exons 14–16 of *AGTPBP1* was amplified by PCR reaction using KAPA2G Fast HotStart Kit (KAPA Biosystems) with forward primer: 5′-TCAGAGGTGGCTTATCCCGA-3′ and reverse primer: 5′-GAATCCACCATGGTCTGGCA-3′. The resultant fragments were separated by 2% (w/v) agarose gel electrophoresis and their sequence determined by Sanger sequencing.

Cloning for separation of alleles

RNA and cDNA were prepared as above. The following primers were used to amplify an amplicon spanning both variants and to integrate an EcoRI restriction site: forward primer: 5′-taagcagaattcCACCATTCCAGAAGAGGGAG A-3′ and reverse primer: 5′-taagcagaattcGACTTGGACT TTGCCACTGC-3′. The amplicon and pCS2+ vector were cut using EcoRI restriction enzyme and were ligated using T4 ligase. Clones were amplified by PCR and Sanger sequenced using the following primers: forward primer: 5′-CACCATTCCAGAAGAGGGAGA-3′ and reverse primer: 5′-GACTTGGACTTTGCCACTGC-3′.

Results

Clinical reports

Clinical data is summarized in Table 1.

Family 1 (Fig. 1a, individual III-1)

The proband is a female firstborn child to healthy Palestinian Muslim Arab parents who are first cousins. She was born after 41 weeks of gestation by spontaneous vaginal delivery. Birthweight was 3280 g, head circumference was 35 cm (50th percentile for age) and length was 50 cm (50th percentile for age). After birth she was noted to be hypotonic and have a weak cry, necessitating temporary respiratory assistance. Apgar score was 7 at 1 min and 9 at 5 min. At the age of 2 months she continued to have weak sucking, axial hypotonia, poor head control and hyporeflexia, and was referred for metabolic and genetic assessment. Thyroid function tests, creatine kinase, ammonia, and

Table 1 Clinical features of affected individuals

Clinical feature	Family 1, Individual III-1	Family 2, Individual II-6
Age at last evaluation	15 months	4 years 2 months
Gender	F	M
Ethnicity	Palestinian, first cousin parents	Ashkenazi Jewish
Gestational week	41	39
Birthweight	3280 g (AGA)	3500 g (AGA)
Apgar scores (1/5 min)	7/9	NA
Respiratory distress	+ (tracheostomy)	+
Mechanical ventilation	+ (continuous since 6 months)	Non-invasive mechanical ventilation at night (since 3 years 8 months); oxygen supplementation during the day
Mechanical insufflation-exsufflation device	+	+
Feeding difficulties	+ (gastrostomy)	+ (gastrostomy)
Recurrent aspirations; recurrent pneumonia	+	+
Poor head control	+	+
Axial hypotonia	+	+
Hyporeflexia	+	+
Hearing	Normal	Normal
Ophthalmological exam	Normal	Normal
Lactate	Normal	NA
Creatine kinase	Normal	Normal
Progressive microcephaly	+	+
MRI	Enlarged CSF spaces, small cerebellar hemispheres and vermis	Small cerebellum
Electrophysiology	Severe motor neuropathy (anterior horn cell disease)	NA
Other	−	Hand tremor, tongue fasciculations
AGTPBP1 variants	c.2342+2T>G [hom]	c.2351A>G; p.Tyr784Cys [het]; c.2998C>T; p.Arg1000Ter [het]

ABR auditory brain response, *AGA* appropriate for gestational age, *CMA* chromosomal microarray, *het* heterozygous, *hom* homozygous, *NA* not available

lactate levels were within normal limits. Auditory brain response was normal as was an ophthalmological examination. Brain MRI at 5 months of age showed enlarged CSF spaces, especially in the posterior fossa, with a relatively small volume of cerebellar hemispheres and vermis (Fig. 2a). Spinal muscular atrophy (SMA) due to deletion of the *SMN1* gene was ruled out. Chromosomal microarray (CMA) revealed a normal female molecular karyotype with multiple homozygous regions. The child was hospitalized at age 4 months due to feeding difficulty, choking and failure to thrive and underwent insertion of a feeding gastrostomy. Respiratory distress necessitated intermittent assisted ventilation due to recurrent atelectasis. At 6 months of age, the child underwent insertion of a tracheostomy. Despite her neuromuscular difficulties, the child was alert and communicative. A progressive decrease in head circumference was noted (Fig. S1), with most recent head circumference measuring 43.2 cm at age 15 months (−2 SD).

Electrophysiological examination at 8 months of age revealed severe motor neuronopathy in both lower limbs and left arm (absent CMAPS, no motor responses with high stimulation). Sensory studies showed normal sensory nerve action potentials (SNAPS) for age (Table S1). Needle electromyography (EMG) showed widespread prominent neurogenic findings: active denervation 3–4/4+ fibs & psw's in both tibialis anterior and left posterior deltoid muscles, with complex repetitive discharges (CRDs) and myotonic discharges in some muscles, and reduced motor unit potential (MUP) recruitment in right tibialis anterior, right iliopsoas and left posterior deltoid muscles, giant MUPs (10 mV) in right iliopsoas, and no volition in the left tibialis anterior muscle. Repetitive stimulation was unobtainable due to absent responses in all motor nerves. In

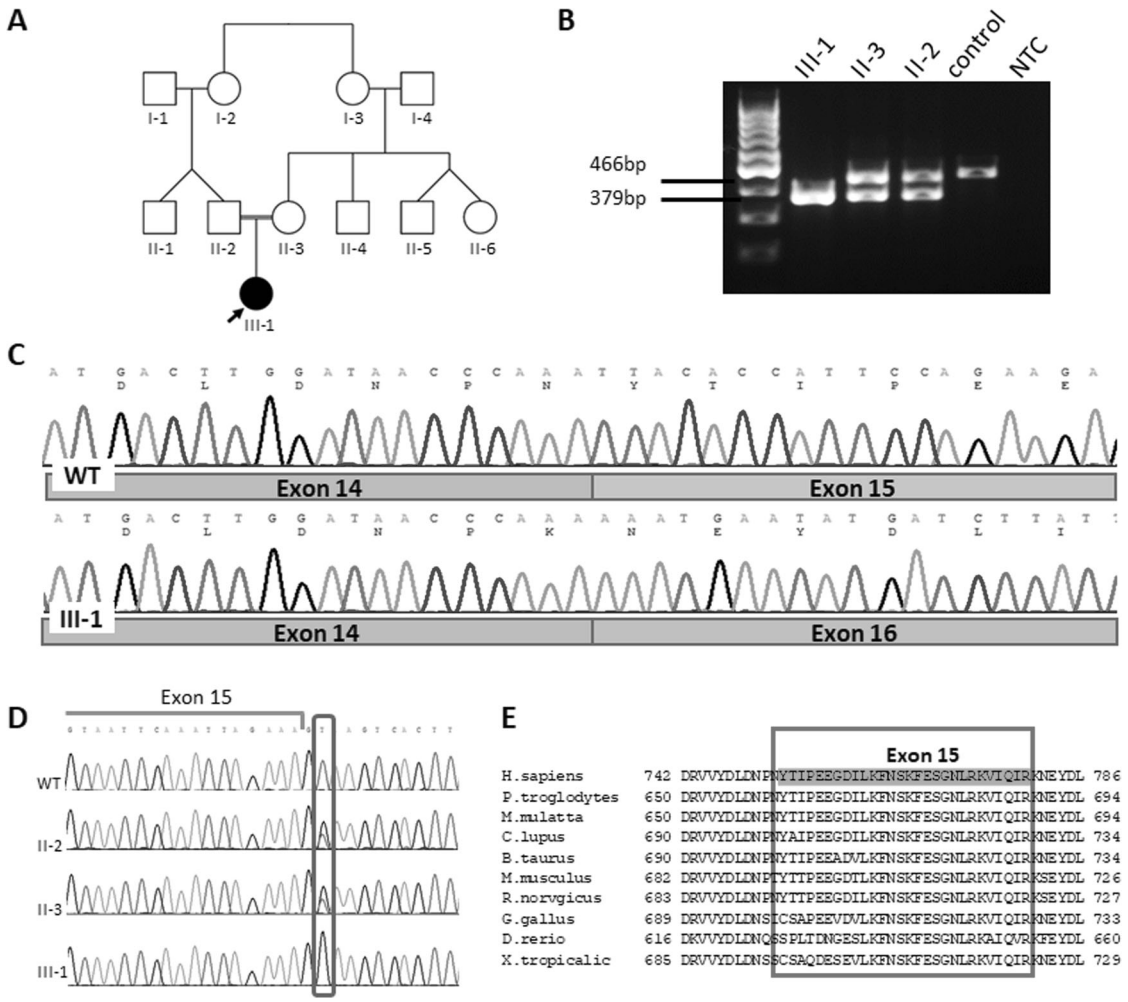

Fig. 1 Pedigree and molecular analysis of Family 1. **a** Pedigree indicating the affected child of first cousin parents. **b** PCR product amplified from cDNA of the proband (III-1), her parents (II-2 and II-3) and a healthy control. Last lane is the no-template control (NTC). **c** Sanger sequencing of cDNA from healthy control (upper panel) and individual III-1 (lower panel) showing skipping of exon 15 as a result of the c.2342 + 2T>G splice site variant. **d** Sanger sequencing of DNA from healthy control, parents and affected individual III-1, showing heterozygosity and homozygosity of the parents and child (correspondingly) to the identified variant. **e** Amino acids encoded by the skipped exon 15 are highly conserved throughout evolution

conclusion, the electrophysiological studies indicated a severe motor neuronopathy with widespread severe active denervation with late MUP recruitment and late chronic reinnervation, and normal sensory studies. These findings were compatible with anterior horn cell disease.

Family 2 (Fig. 3a, individual II-6)

The proband is a 50-months-old-male, the youngest child of 6, born to healthy unrelated Ashkenazi Jewish parents after 39 weeks of gestation, at a birth weight of 3500 g. The perinatal course was uneventful. At 4 months of age, increasing difficulty in raising his head was noted and he began physiotherapy. At 6 months of age he could track faces and had a social smile though head control was poor and he could not roll over. Tremor of the hands was seen both in movement and at rest and he could not reach for objects. There were decreased movements in the lower limbs. No dysmorphic features were noted. Tongue fasciculations were evident. Muscle tone was low and deep tendon reflexes were decreased. Muscle mass seemed adequate. Further review of systems and physical examinations did not reveal other abnormalities. Brain MRI at the age of 1 year demonstrated cerebellar hypoplasia or atrophy (Fig. 2b). Over the ensuing two years further neurological regression was noted, with inability to purposely move his limbs hands and a decrease in eliciting sounds with minimal capability of communication. He had not achieved milestones such as sitting or standing or talking. Repeated aspirations due to difficulty in eating solid food and slow weight gain led to gastrostomy insertion at two years of age. A progressive decline in the head circumference percentile

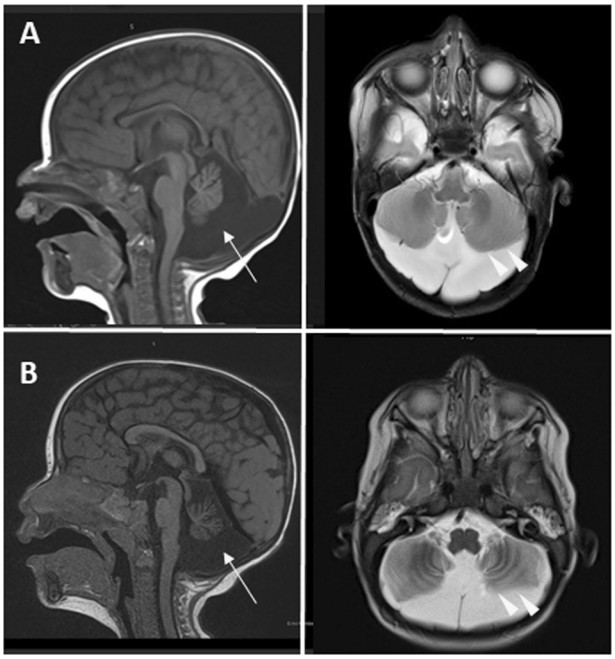

Fig. 2 Brain magnetic resonance imaging (MRI) of affected individuals. Sagittal T1 and axial T2 views demonstrating enlarged CSF spaces, especially in the posterior fossa, with relatively small cerebellar hemispheres and vermis (yellow arrows and arrowheads). **a** Family 1, individual III-1 at 5 months of age. **b** Family 2, individual II-6 at 12 months of age

for age was noted (Fig. S1), and at last examination at 45 months of age was measured to be 46 cm (−2.7 SD). Eyesight and hearing were normal.

Whole exome analysis identified biallelic variants in *AGTPBP1*

Whole exome analysis did not reveal any rare variant in a known gene associated with cerebellar hypoplasia or motor neuropathy. However, patient 1 was homozygous for a splice site intronic variant in the *AGTPBP1* gene chr9: g.88234128A>C [hg19]; NM_001286715.1: c.2342+2T>G; r.2256_2342del. This variant was not found in the GnomAD database nor in the in-house database of ~3500 exomes (Table S2). Parents were both heterozygotes for the variant, and no siblings were available for segregation (Fig. 1a). cDNA generated from the patient blood cells was abnormally short due to skipping of exon 15 (Fig. 1b–d). Exon 15 consists of 87 bp and its loss is predicted to result in the in-frame absence of 29 highly conserved amino acids (Fig. 1e). In addition to the above variant, a homozygous variant in *NCAPD3* (c.1031A>T; p.Glu344Val) was identified (Table S2). Variants in *NCAPD3*, which encodes a condensin complex subunit, have been associated with autosomal recessive primary microcephaly [MIM 617984]. We cannot rule out a potential contribution of this variant to the phenotype although *AGTPBP1* seems to be the main contributor.

In patient 2, exome sequencing identified two variants in the *AGTPBP1* gene (Fig. 3a, b): c.[2351A>G];[2998C>T]. The first was a maternally inherited variant (chr9: g.88234038T>C [hg19]; NM_001286715.1; c.2351A>G, p.Tyr784Cys), affecting a highly conserved residue (Fig. 3c), and the second was a de novo stopgain variant predicted to cause a premature termination codon (chr9: g.88203274G>A [hg19]; NM_001286715.1; c.2998C>T, p.Arg1000Ter). Paternity was confirmed by short tandem repeats (STR) analysis. Neither variant was found in the GnomAD database nor in the in-house database (Table S2). Segregation studies revealed the c.2351A>G allele in heterozygous state in the mother and three sisters of the proband (Fig. 1a), while no siblings shared the de novo c.2998C>T variant (Fig. 3a).

To determine whether the de novo allele was located in trans to the inherited maternal allele, a fragment encompassing both variants was amplified from cDNA and cloned to separate the alleles. Clones were sequenced by Sanger, and either showed (a) wild-type allele at position r.2351a>g and variant allele at position r.2998c>u, or (b) vice versa, variant allele at position r.2351a>g and wild-type allele at position r.2998c>u (Fig. 3d). This confirmed that the variants were on two separate alleles, i.e., in trans position. Notably, the r.2998c>u variant allele leading to a premature termination codon was seen in only 1 of 15 clones sequenced, indicating significant nonsense mediated decay of the relevant transcript.

Discussion

In this study, we report two unrelated individuals with congenital muscle weakness progressing to flaccid tetraparesis, muscle wasting and areflexia. Electrophysiological studies performed in the affected individual in Family 1 were indicative of anterior horn cell disease, and brain neuroimaging showed decreased cerebellar volume. The combination of motor neuropathy and cerebellar hypoplasia/atrophy is suggestive of pontocerebellar hypoplasia type 1 (PCH1). As mentioned above, PCH1 demonstrates locus heterogeneity, and has been associated with variants in genes encoding several exosome components, genes involved in amyloid-β precursor protein (APP) maintenance and a mitochondrial dynamics protein.

Exome sequencing in both patients revealed biallelic variants in *AGTPBP1*. The first patient had a splice-site variant leading to exon skipping, while the second patient had an inherited variant and a second de novo variant, a rare mechanism for autosomal recessive disease. The encoded protein, ATP/GTP-Binding Protein 1 (OMIM *606830), also known as NNA1 (Nervous system Nuclear protein induced by Axotomy) and CCP1 (Cytosolic

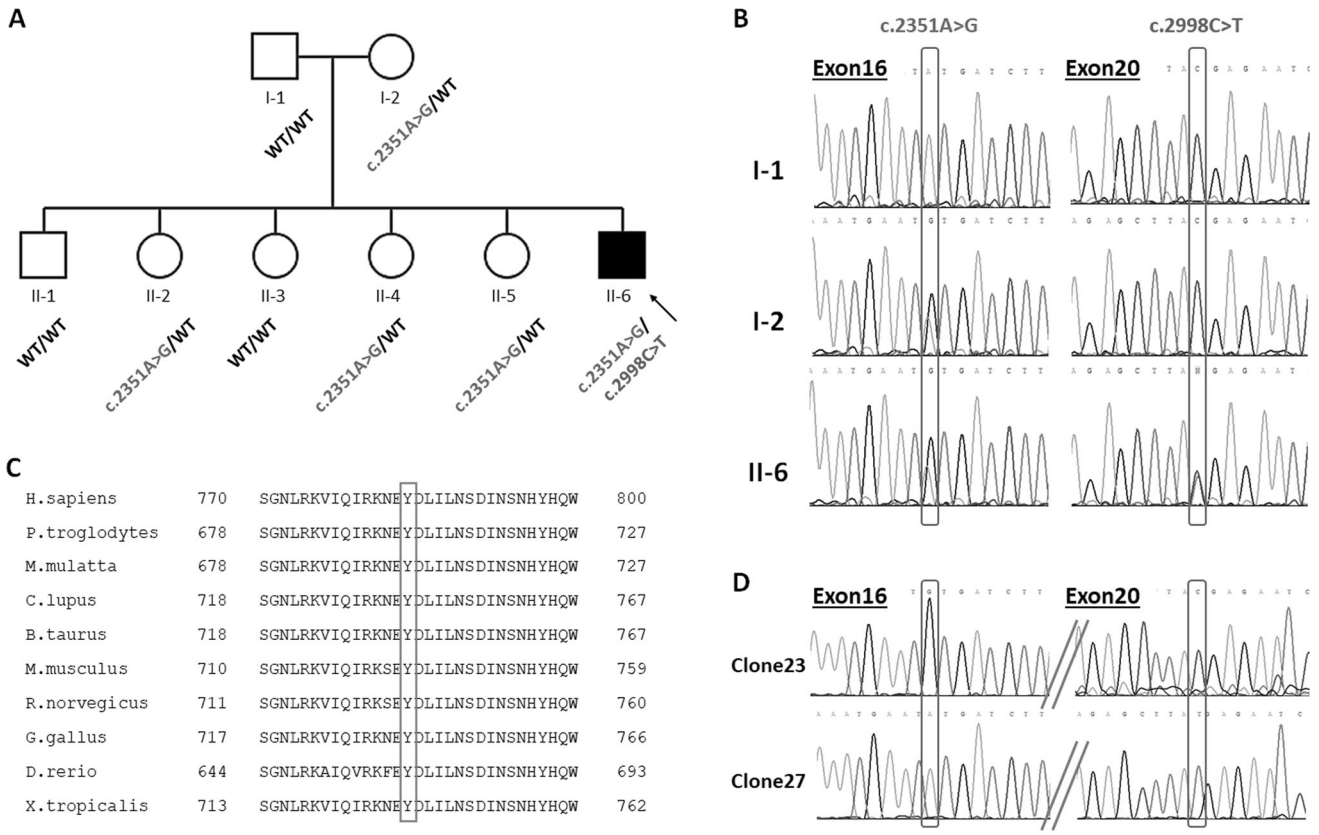

Fig. 3 Pedigree and molecular analysis of Family 2. **a** Pedigree indicating the affected child (c.[2351A>G]; [2998C>T] and genotypes of family members. The c.2351A>G variant is maternally inherited, while c.2998C>T is de novo. **b** Sanger sequencing of DNA from parents and affected individual 2. **c** Tyr784 and neighboring amino acids are highly conserved throughout evolution. **d** Allele separation by cloning of a single amplicon of exons 15–21 from cDNA, followed by Sanger sequencing, indicated that the inherited and *de novo* variants were *in trans* position. The upper panel (clone 23) shows the variant allele in exon 16 and the wild-type allele in exon 20, while the lower panel (clone 27) shows the wild-type allele in exon 16 and the variant allele in exon 20

Carboxypeptidase 1), catalyzes deglutamylation of polyglutamylated proteins [18]. The protein consists of an ATP/GTP-binding motif of the P-loop type, a leucine zipper, a nuclear localization signal, a zinc carboxypeptidase signature, and a nucleotide-binding site. It contains multiple canonical phosphorylation sites for protein kinase C, casein kinase II, and cGMP/cAMP-dependent kinases. In addition, it harbors four consensus phosphorylation sites for tyrosine kinases [19].

Polyglutamylation is a post-translational modification in which glutamate side chains of variable lengths are formed on the modified protein. It is evolutionarily conserved from protists to mammals and its most prominent substrate is tubulin [20, 21]. Polyglutamylated microtubules which accumulate during brain development are involved in synapse vesicle transport or neurite outgrowth through interactions with motor proteins or microtubule-associated proteins, respectively. AGTPBP1 specifically catalyzes the removal of the penultimate glutamate residue from detyrosinated α-tubulin, thus generating Δ2-tubulin. In addition, it shortens posttranslationally generated glutamate side chains on tubulin, serving as a tubulin deglutamylase. AGTPBP1 also removes glutamic acids from the C termini of additional substrates, including myosin light chain kinase 1 (MLCK1) [18].

Variants in *AGTPBP1* were previously reported in the mouse Purkinje cell degeneration (*pcd*) phenotype [21, 22]. Tubulin polyglutamylation was highly increased specifically in the degenerated brain areas, suggesting that microtubule hyperglutamylation is directly linked to neurodegeneration. Downregulating polyglutamylation partially prevented neurodegeneration [23].

Additional spontaneously occurring *AGTPBP1* mutants are the New Zealand Romney lambs. These animals were reported 20 years ago because of their lower motor neuron-like disease manifesting by progressive weakness and tetraparesis which started after the first week of life [24]. Predominant histological lesions were degeneration and loss of neurons in the ventral horns of the spinal cord and the brain stem, Wallerian degeneration of the motor nerves and denervation atrophy of skeletal muscles fibers. Of note, cerebellar histology was intact at 4 weeks of age.

Subsequent molecular analysis disclosed a homozygous variant in the sheep ortholog *AGTPBP1*, affecting a highly conserved residue in the catalytic domain of the encoded protein [25]. A homozygous variant (p.Arg970Trp) was previously reported in a human child with global developmental delay and brain atrophy [26], yet no further details were available.

Thus our data suggest that biallelic variants in *AGTPBP1* are associated with PCH and SMA-like disease; this phenotype is reminiscent of the Purkinje cell degeneration (*pcd)* mouse and the motor-neuron disease in the New Zealand Romney sheep [21, 24]. Most of our patients' symptoms likely derived from Purkinje cell degeneration and motor neuronopathy. We propose that these degenerative processes result from impaired deglutamylation of central nervous system (CNS) specific tubulin. Interestingly, *AGTPBP1* was identified as the most significant gene coexpressed with C9orf72, a gene implicated in familial and sporadic amyotrophic lateral sclerosis and frontotemporal dementia, and may serve as a *C9orf72* interacting partner in regulation of neuronal function within the CNS [27]. However, additional studies are required in order to elucidate the underlying pathogenic mechanism of *AGTPBP1*-related disease.

The treatment of affected individuals with *AGTPBP1*-related disease is at present symptomatic and supportive. Presence of robust animal models for this complex disorder may assist research for therapeutic options such as down-regulating polyglutamylation in affected individuals.

Accession numbers

The ClinVar accession numbers for the DNA variant data reported in this manuscript are SCV000844952, SCV000844953, and SCV000864167.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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