Novel CAPN1 missense variants in complex hereditary spastic paraplegia with early-onset psychosis

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Introduction

The hereditary spastic paraplegias (HSPs) are a group of more than 80 different neurogenetic disorders. While pure and complex forms share the clinical feature of progressive spasticity due to corticospinal tract dysfunction, complex HSP presents with additional neurological symptoms such as cognitive impairment, ataxia, neuropathy, seizures, and psychiatric symptoms. In some cases, nonmotor symptoms precede the onset of motor symptoms and signs of corticospinal tract dysfunction can be subtle initially. Next-generation sequencing has enabled the interpretation of molecular findings and can support a diagnosis. SPG76 is a complex form of HSP, caused by biallelic mutations in CAPN1, encoding the calcium-activated protease calpain-1. Heterozygous variants in RCL1 are associated with early-onset psychosis. Here we further characterize a patient with novel CAPN1 variants and a previously reported heterozygous stop-gain mutation in RCL1, who presented in adolescence with psychiatric symptoms, later followed by lower limb spasticity, cerebellar signs, and neurogenic bladder dysfunction.

Methods

Clinical characterization

This study was approved at the Boston Children’s Hospital (IRB-P00033016). Written consent was obtained. Trio-exome sequencing was performed at GeneDx (Gaithersburg, MD, USA).

In silico analyses

Information on the human CAPN1 protein sequence was obtained from the Universal Protein Resource (ID: P07384) and AlphaFold Protein Structure (DeepMind Technologies) databases, as well as published data. Protein tertiary structures were visualized using Pymol (v2.5.0, Schrödinger, LLC, New York, NY, USA). CADD PHRED scores (v1.6) of all possible base substitutions of the CAPN1 transcript were computed and mapped to the corresponding protein sequence. All variants were harmonized to NM_001198868.2/GRCh38/hg38. Modeling of the CAPN1 primary protein structure and variants was done in R version 4.1.0 (2021-05-18) and RStudio (version 1.4.1103; RStudio, Inc., Boston, MA, USA).

Functional assays

Primary skin fibroblasts were cultured according to established protocols. Calpain enzyme activity was assessed using a fluorogenic enzyme assay (Sigma-Aldrich, St. Louis, MO, USA; Cat#QIA120). Calpain activity was quantified in two biological replicates and normalized to protein concentrations. Western blotting was performed as described, and each experiment was performed in at least three biological replicates. Antibodies and reagents are listed in Table S1. For treatment with PHLPP1 inhibitor fibroblasts were seeded at 500 × 10^3 in 6-well-plates and 24 h postplating media containing NSC117079 or 0.5% DMSO was added. After 24 h of treatment, cells were harvested. Statistical analysis was performed with Prism (GraphPad Software, San Diego, CA, USA). Groups were compared using Mann–Whitney U test or two-way ANOVA with Tukey’s post hoc analysis. p < 0.05 was considered significant.

Clinical Presentation

The proband’s initial presentation has been described by Brownstein et al. Briefly, at age 14-years the proband presented with subacute-onset catatonia, and several months of anxiety, auditory and visual hallucinations, disorganized thoughts, paranoia, and aggression, requiring hospitalization. Prior to this, there had been no neurological or psychiatric concerns. He was born at full term to nonconsanguineous parents of mixed European ancestry, achieved all developmental milestones appropriately, and was an average student with no history of learning difficulties. Given the above constellation of symptoms, he underwent a broad diagnostic evaluation. Neurological examination at age 14-years was negative for pyramidal or cerebellar signs. EEG and brain MR imaging showed no significant abnormalities. CSF studies were notable for a persistently elevated protein (initially 80 mg/dL, remained between 50 and 65 mg/dL on repeat measurements), but serum and CSF autoantibody panels were negative. He received immunomodulatory treatment for presumed antibody-negative autoimmune encephalitis without improvement. His psychiatric symptoms stabilized on treatment with clozapine. Serial neuropsychological examinations showed deficits in attention and executive function. At age 15-years, he developed urinary urgency and incontinence. Clozapine can lead to urinary incontinence and may have been contributing to these symptoms, however, urodynamical studies confirmed neurogenic bladder dysfunction. At the age of 16-years, his gait was noted to decline, he complained about muscle cramps and his legs being stiff and started having difficulties with handwriting. At the age of 18-years, he was found to have an intention tremor.
of the hands, mild spasticity in his distal legs, and a positive Babinski sign. At age 20 years, his exam was notable for dysarthria and a spastic gait. Between the age of 16 and 20 years, his spastic paraplegia was found to have progressed leading to a Spastic Paraplegia Rating Scale score of 10 at last follow-up.

**Molecular Findings and In Silico Analyses**

Trio-exome-sequencing demonstrated two novel missense variants in *CAPN1* and a paternally inherited heterozygous stop-gain mutation in *RCL1* (Table 1). The paternally inherited *CAPN1* variant (c.1712A>G (p.Asn571Ser)) showed an allele frequency of 0.000017 in gnomAD exomes with a homozygous allele count of 0 and is predicted to result in a conservative amino acid change in the penta-EF-hand (PEF) Ca$^{2+}$ binding domain. The paternally inherited *CAPN1* variant (c.1991C>T (p.Ser664Leu)) showed an allele frequency of 0.000012 and a homozygous allele count of 0 and leads to a nonconservative amino acid exchange also affecting the PEF domain. CADD PHRED scores were 27.7 and 24.1, respectively. Analysis of sequence conservation suggests the c.1712A>G variant to be highly and the c.1991C>T variant to be moderately conserved (Table 1). No tertiary structure has been published for the PEF domain of human calpain-1. We, therefore, used the AlphaFold Protein Structure Database$^{12,13}$ to predict interactions of the human calpain-1. We, therefore, used the AlphaFold Protein Structure Database$^{12,13}$ to predict interactions of the Asn571 and Ser664 residues. Asn571 is predicted to form two hydrogen bonds with Phe585 and Ser664 forms a hydrogen bond with Ile660. Based on these predictions, amino acid exchanges at positions 571 and 664 likely lead to a change in tertiary structure (Fig. 1A). An additive model of CADD PHRED scores for all possible missense across the linear protein structure identified no clear mutational hotspots with low tolerability to missense variation across the entire protein (Fig. 1B). The *RCL1* variant has been discussed previously.$^5$

**Functional Assessment of Novel *CAPN1* Variants**

Considering the possible pathogenicity of the *CAPN1* missense variants implied by in silico predictions and their localization in the PEF domain, we next performed experiments in patient-derived fibroblasts to examine the impact on calpain-1 activity and downstream signaling. Figure 2A shows an overview of the calpain pathway. Pan-calpain activity was reduced by 30.4 ± 11.5% in the proband’s fibroblasts compared to the control (Fig. 2B). To determine whether this was caused by altered protein abundance, levels of calpain-1 and calpain-2 were quantified, with no changes identified (Fig. 2C). Next, we measured protein levels of the calpain-1 substrate PHLPP1 and the calpain-2 substrate PTEN.$^{14,15}$ PHLPP1, but not PTEN, was significantly increased in the proband’s cells (Fig. 2D and E). PHLPP1 is an important negative regulator of the Akt signaling pathway through specific dephosphorylation of the active phospho-Akt at Ser473 (pAkt) (Fig. 2A).$^{16}$ In agreement with increased PHLPP1 levels present in the proband’s cells, levels of pAkt were reduced (Fig. 2F). To further investigate whether this was a result of increased PHLPP1 activity, we treated fibroblasts with increasing concentrations of NSC117079, a potent inhibitor of PHLPP1, and determined pAkt levels after 24 h of treatment. Treatment with 50 μmol/L NSC117079 increased pAkt in the proband’s fibroblasts (36.2% vs. 23.4% in the control, p = 0.14, Fig. 2G). Taken together, these results provide evidence for a partial loss of calpain-1 function in the proband’s cells, leading to disinhibition of PHLPP1 activity and subsequent reduction of pAkt levels. This supports a diagnosis of *CAPN1*-related SPG76.

**Table 1.** Variants discovered in the proband.

| Gene | Genomic location (hg38) | Inheritance | Variant impact | ACMG classification | Conservation prediction (GERP/PhyloP100way/PhastCons100way) | Allele count gnomAD (homozygous/total) | Allele frequency gnomAD (exomes/ genomes) |
|------|-------------------------|-------------|----------------|--------------------|----------------------------------------------------------|-------------------------------------------|------------------------------------------|
| *CAPN1* | NM_000011.10: g.64975716A>G | Paternal | Missense, conservative amino acid exchange | Likely pathogenic | 5.23/9.318/1.000 | 0/4 | f = 0.0000167/0.0000319 |
|        | NM_001198868.2: c.1712A>G | Paternal | Missense, conservative amino acid exchange | Likely pathogenic | 4.21/7.555/0.961 | 0/3 | f = 0.0000121/0 |
|        | NP_001185797.1: p.Asn571Ser | Paternal | Missense, conservative amino acid exchange | Likely pathogenic | 5.92/7.542/1.000 | 0/0 | f = 0/0 |

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Discussion

We here report a patient with significant psychiatric symptoms during adolescence and subsequent development of a spastic gait, intention tremor and neurogenic bladder dysfunction. The proband was previously reported as an index case for RCL1-associated psychiatric syndrome \(^5\),\(^17\) but his progressive motor and urinary symptoms remained unexplained. Exome sequencing showed compound-heterozygous missense variants in \(\text{CAPN1}\). Calpain-1 is an important regulator of neuronal survival, axonal homeostasis and synaptic plasticity,\(^18\) and loss of calpain-1 function is known to cause SPG76.\(^4\) In light of the proband’s evolving neurological syndrome, consistent with a complex form of HSP, we pursued functional assays to assess calpain-1 function in his cells.

Our in silico analyses predicted a potential impact of both \(\text{CAPN1}\) variants on protein tertiary structure. In support of this, we found that, although our patient’s \(\text{CAPN1}\) variants do not alter protein expression and stability, the resultant amino acid exchanges in the PEF domain lead to reduced enzyme activity. This results in reduced degradation of PHLPP1 and subsequently reduced Akt-signaling. Further studies are needed to separate the impact of each variant on enzyme activity. Another important consideration are cell-type-specific differences in the calpain-1 pathway. Although primary fibroblasts are an important tool for functional studies, patient-derived neuronal cells will provide a more relevant model system to understand the molecular mechanisms of \(\text{CAPN1}\)-associated HSP.

Both RCL1 haploinsufficiency and bi-allelic variants in \(\text{CAPN1}\) have been associated with a variety of psychiatric symptoms.\(^5\),\(^6\),\(^19\) SPG76 shows a wide phenotypic spectrum with pure and complex forms and significant inter- and intra-familial variability.\(^4\),\(^6\),\(^20\)–\(^23\) Our patient’s current
symptoms and age at presentation align with the reported spectrum of SPG76 while his early-onset psychosis may be attributable to the additional effect of his RCL1 variant. Further studies are needed to evaluate if psychiatric symptoms precede motor symptoms in other patients with SPG76 and if RCL1 variants can act as a
In conclusion, we reported a patient with CAPN1-associated SPG76 and expand the clinical and molecular spectrum associated with this rare form of complex HSP. The present case highlights the need to carefully characterize the functional impact of novel variants and that multiple genes can contribute to complex neuropsychiatric diseases.

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Conflict of Interest

D. E. F. received a speaker honorarium from the Movement Disorder Society, publishing royalties from the Cambridge University Press and reports research funding through a joint research agreement with Astellas Pharmaceuticals Inc.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primary antibodies and reagents.