




RESEARCH ARTICLE

Novel *CAPN1* mutations extend the phenotypic heterogeneity in combined spastic paraplegia and ataxia

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Abstract

Objective: Recessive mutations in the *CAPN1* gene have recently been identified in spastic paraplegia 76 (SPG76), a complex hereditary spastic paraplegia (HSP) that is combined with cerebellar ataxia, resulting in an ataxia-spasticity disease spectrum. This study aims to assess the influence of *CAPN1* variants on the occurrence of SPG76 and identify factors potentially contributing to phenotypic heterogeneity. **Methods:** We screened a cohort of 240 unrelated HSP families for variants in *CAPN1* using high-throughput sequencing analysis. We described in detail the clinical and genetic features of the SPG76 patients in our cohort and summarized all reported cases. **Results:** Six unreported *CAPN1*-associated families containing eight patients with or without cerebellar ataxia were found in our cohort of HSP cases. These patients carried three previously reported homozygous truncating mutations (p.V64Gfs*103, c.759+1G>A, and p.R285*), and three additional novel compound heterozygous missense mutations (p.R481Q, p.P498L, and p.R618W). Lower limbs spasticity, hyperreflexia, and Babinski signs developed in about 94% of patients, with ataxia developing in 63% of cases. In total, 33 pathogenic mutations were distributed along the three reported functional domains of calpain-1 protein, encoded by *CAPN1*, with no hotspot region. A comparison of gender distribution between the two groups indicated that female SPG76 patients were significantly more likely to present with complicated HSP than male patients ($P = 0.015$). **Interpretation:** Our study supports the clinically heterogeneous inter- and intra-family variability of SPG76 patients, and demonstrates that gender and calpain-1 linker structure may contribute to clinical heterogeneity in SPG76 cases.

Introduction

Hereditary spastic paraplegia (HSP) comprises a group of clinically heterogeneous disorders characterized by progressive spasticity and hyperreflexia of the legs due to corticospinal tract degeneration.^{1,2} Historically, HSP is distinguished as pure or complicated forms on the basis of clinical grounds.³ Pure form HSP shows predominant spasticity and weakness restricted to the lower limbs, and often presents hypertonia, hyperreflexia, and Babinski sign. In addition, bladder dysfunction and sensory disturbance are also frequently reported in the pure form. Complicated form HSP is accompanied by additional

neurological or extra-neurological signs, including ataxia, intellectual disability, peripheral neuropathy, amyotrophy, cataracts, hypopigmentation, and others.

HSP is also a genetically heterogeneous disease. To date, more than 82 known or suspected genes or genetic loci have been reported to cause HSP.⁴ Notably, it has been proposed that many autosomal recessive HSP genes remain unidentified.³ Recently, loss-of-function mutations in the *CAPN1* gene have been described as causative for spastic paraplegia 76 (SPG76).⁵ *CAPN1* encodes a neutral calcium-activated protease known as calpain-1 protein, which is involved in processes of synaptic plasticity, neuronal migration, neuronal maintenance and necrosis,

among others.⁶ Calpain-1 contains three functional domains, including a protease domain, a C2-like (C2L) Ca²⁺ binding domain, and a penta-EF-hand (PEF) Ca²⁺ binding domain.^{7,8} Notoriously, HSP patients with autosomal recessive inheritance showed higher clinical complexity, especially due to ataxia resulting in ataxia-spasticity spectrum.⁹ Even though mutations in *CAPN1* were initially associated with cerebellar ataxia in dogs and humans,^{6,10} as well as in human complicated form HSP presented with any of ataxia, dysarthria and amyotrophy,⁵ reports of pure form HSP patients with *CAPN1* mutations are increasing.^{11–24} Given this broad range of symptoms and contributing factors, it remains an ongoing challenge to find a specific, reliable correlation between genotype and phenotype for this disease complex.

In this study, we describe six additional *CAPN1*-associated SPG76 families containing eight affected individuals with or without cerebellar signs. These patients had six homozygous or compound heterozygous variants. In addition, we provide a summary review of SPG76 literature in order to clarify the phenotypic heterogeneity associated with this disease.

Patients and Methods

Subjects recruitment

This retrospective study was carried out at the Department of Neurology of the First Affiliated Hospital of Fujian Medical University. In total, 240 families that fulfilled Fink's diagnostic criteria for HSP symptoms were enrolled irrespective of their genetic diagnosis.^{1,2,25–27} Clinical features, brain MRI findings, and genetic data were collected and analyzed for each available case. The ethics committees of the First Affiliated Hospital of Fujian Medical University approved the study, and written informed consent was obtained from each of the participants.

High-throughput sequencing and data analysis

Genomic DNA was extracted from the peripheral blood of the included subjects using a QIAGEN kit. Prior to high-throughput sequencing (HTS), multiplex ligation-dependent probe amplification (MLPA) was applied to detect the large deletions or copy number variations (CNV) in commonly known pathogenic genes (*ATL1*, *SPAST*, *NIPA1*, *SPG11*, *REEP1*, and *SPG7*) using SALSA MLPA probe mixes (MRC-Holland). Subsequently, point mutations and small indel (insertion/deletion) mutations (< 50 base pairs) in 82 known HSP-causative genes (<https://neuromuscular.wustl.edu>) were screened by

targeted next-generation sequencing (NGS) or whole-exome sequencing (WES) using the Illumina HiSeq 3000 platform.^{25–28} The pathogenicity of the filtered variants was then investigated by *in silico* prediction tools. The variants were finally comprehensively classified according to the American College of Medical Genetics and Genomics (ACMG) criteria.²⁹ Specifically, each pathogenic criterion is weighted as very strong (PVS1), strong (PS1–4), moderate (PM1–6), or supporting (PP1–5). In particular, segregation of the identified pathogenic *CAPN1* variants (NM_001198868.2) was investigated in their available relatives using Sanger sequencing with the variant-specific primers (Supplemental File S1: Table S1).

Statistical analysis

Results are expressed as ratios or means with min to max values. To compute p values, Student's t-test and Chi-squared followed by Bonferroni test were used, as indicated in the figure legends.

Results

Genetic spectra of CAPN1

A total of 110 families carrying the known HSP-causative genes were diagnosed through HTS analysis (Supplemental File S2: Figure S1a). Remarkably, six unrelated families carrying *CAPN1* mutations were identified, accounting for 12.2% (6/49) of the autosomal-recessive HSP cases (Supplemental File S2: Figure S1b). Genetically, six homozygous or compound heterozygous variants of *CAPN1* were detected from the aforementioned six families (Family 1 to Family 6) (Fig. 1A and Supplemental File S2: Figure S2), three of which were previously documented pathogenic homozygous mutations (c.182_183insC (p.V64Gfs*103), c.759+1G>A, and c.853C>T (p.R285*)),^{6,11,21} The other three additional compound heterozygous missense variants ((c.1442G A (p.R481Q), c.1493C>T (p.P498L), and c.1852C T (p.R618W)) occurred at extremely low allele frequency (<7 × 10⁻⁵) and have not yet been reported. The former two of these mutations were located in the C2L Ca²⁺-binding domain, while the latter was found in the PEF Ca²⁺ binding domain of calpain-1 (Fig. 1b). Notably, the three novel variants showed concordant segregation with the HSP phenotype, and were predicted to be deleterious. Thus, these three missense variants can be classified as likely pathogenic according to the ACMG criteria (PM1+PM2+PP1+PP3) (Supplemental File S1: Table S2).²⁹ Intriguingly, two separate, unrelated families (Families 1 and 4) harbored the same homozygous mutation p.V64Gfs*103, while the other two families (Families 2

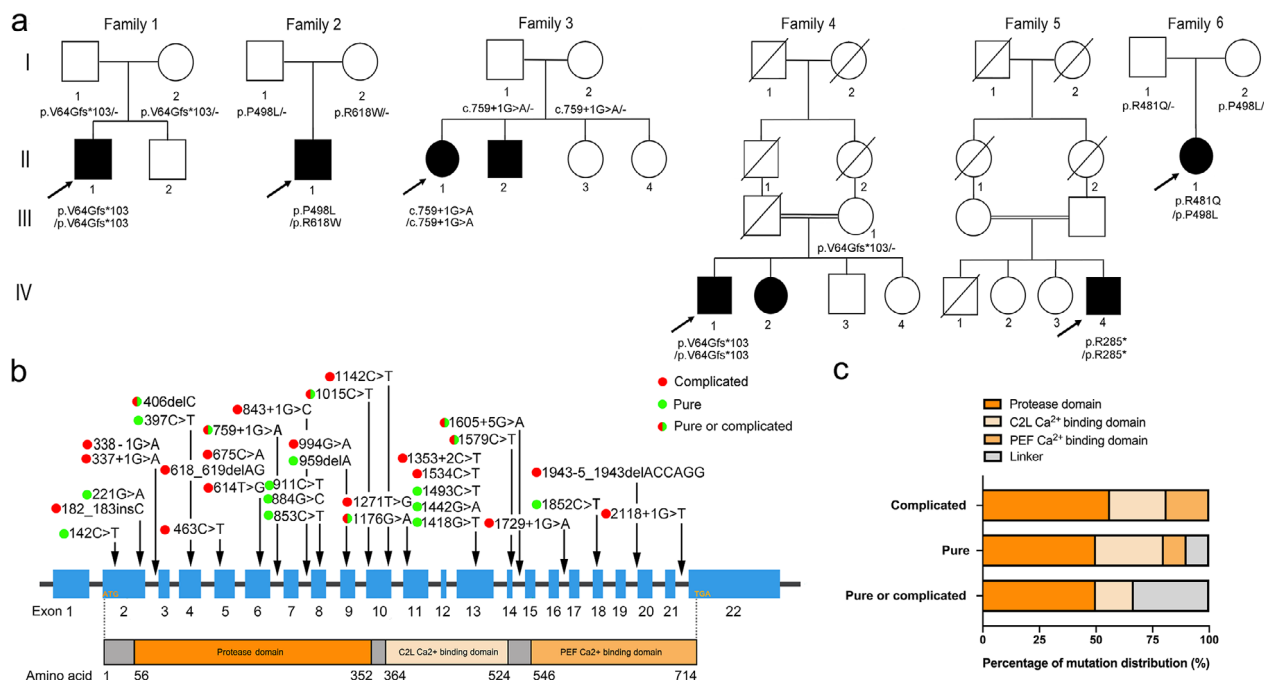


Figure 1. Mutational spectrum of *CAPN1* in SPG76 patients. (A) The pedigrees of six SPG76 families in our cohort, with *CAPN1* mutations indicated. Filled black squares and circles indicate affected individuals. The proband in each family is indicated by an arrow. The genotypes of all available family members were determined. Dash symbol indicates reference allele. (B) The mutation distribution across the *CAPN1* gene and corresponding calpain-1 protein containing a protease domain (amino acid 56-352), a C2L Ca²⁺-binding domain (amino acid 364-524), and a PEF Ca²⁺-binding domain (amino acid 546-714). Gray bars indicate the linker structure. Filled green circles highlight the mutations causing pure form HSP. Filled red circles highlight the mutations causing complicated form HSP. The half-red and half-green circles highlight the mutations causing either pure or complicated form HSP. (C) Statistical analysis of the correlation between mutation distribution and HSP forms.

and 6) carried a separate, identical heterozygous mutation p.P498L.

Clinical profiles of *CAPN1*-related SPG76

The clinical profiles of eight patients from the six *CAPN1*-related SPG76 families are shown in Table 1. Collectively, the mean \pm SD of age at onset of the affected individuals was 27.6 ± 6.3 years, ranging from 19- to 38-years-old. The mean \pm SD of disease duration was 12.4 ± 6.0 years. Clinically, bilateral lower limb spasticity, with or without weakness, was the initial symptom for all cases. Moreover the brisk deep tendon reflex observed in four extremities and extensor plantar responses were the common clinical signs among all eight patients.

Three probands (II-1 in Family 2, IV-4 in Family 5, and II-1 in Family 6) had the core features of HSP without cerebellar signs, and were considered as prototypical pure forms. Five other affected individuals (II-1 in Family 1, II-1 and II-2 in Family 3, and IV-1 and IV-2 in Family 4) exhibited ataxia and hypermyotonia in the lower limbs, without the dysdiadochokinesia, nystagmus, or slurred speech. Specifically, the male patient II-2 in Family 3 first

noticed symptoms of slowly progressive lower limb stiffness and unsteadiness at the age 38. He presented with truncal and gait ataxia, but was still able to walk independently without aids. His cranial MRI revealed very mild vermian atrophy. The patients IV-1 and IV-2 in Family 4 were born to the third-degree consanguineous parents and started with gait difficulty at 21 years. In these two patients, dysarthria began approximately 20 years later, with their ability to walk without aid lost by age 40. Notably, none of the affected individuals complained of bladder dysfunction or any sensory abnormalities. However, the cranial MR imaging of the other available individuals showed no remarkable signs of disease.

Comprehensive analysis of phenotypic heterogeneity for *CAPN1*-associated SPG76

The combination of our cohort with the other previously reported cases resulted in a total of 35 pedigrees, which included 67 SPG76-affected individuals that carried a total of 33 homozygous or compound heterozygous *CAPN1* mutations among them.^{5,6,11-24} These mutations were distributed along the entire *CAPN1* locus with no

Table 1. A summary of clinical features of eight affected individuals in six families with CAPN1 mutations

	Family 1		Family 2		Family 3		Family 4		Family 5		Family 6	
	II-1	II-1	II-1	II-1	II-1	II-2	IV-1	IV-2	IV-4	IV-1	II-1	
Mutation	c.182_183insC	c.1493C>T and c.1852C>T	c.1852C>T	c.759+1G>A	c.759+1G>A	c.759+1G>A	c.182_183insC	c.182_183insC	c.853C>T	c.853C>T	c.1442G>A and c.1493C>T	
Gender	Male	Male	Female	Male	Female	Male	Male	Female	Male	Male	Female	
Age at onset, year	27	33	33	38	33	38	21	21	28	28	19	
Disease duration, year	14	9	11	4	11	4	20	19	18	18	4	
Disability score	3	2	2	2	2	2	3	3	2	2	2	
Initial symptom	Weakness and stiffness of legs	Weakness and stiffness of legs	Stiffness of legs	Stiffness of legs	Stiffness of legs	Stiffness of legs; unsteadiness	Stiffness of legs	Stiffness of legs	Weakness and stiffness of legs	Weakness and stiffness of legs	Stiffness of legs	
Dysarthria	-	-	-	-	-	-	+	+	-	-	-	
Cerebellar sign	Limb ataxia	-	Truncal and gait ataxia	Truncal and gait ataxia	Truncal and gait ataxia	Truncal and gait ataxia	Limb ataxia	Limb ataxia	-	-	-	
Hypermyotonia-UL	+	+	-	-	-	-	+	+	-	-	-	
Hypermyotonia-LL	+	+	+	+	+	+	+	+	+	+	+	
Hyperreflexia-UL	+	+	+	+	+	+	+	+	+	+	+	
Hyperreflexia-LL	+	+	+	+	+	+	+	+	+	+	+	
Lower limb weakness	+	+	-	-	-	-	-	-	+	+	-	
Extensor plantar response	+	+	+	-	-	-	+	+	+	+	+	
Sensory impairment	-	-	-	-	-	-	-	-	-	-	-	
Brain MRI	Normal	Normal	NA	Mild cerebellar atrophy	Normal	Normal	Normal	Normal	Normal	Normal	Normal	

Disability score: 1 = normal, 2 = walks but cannot run, 3 = walks with aids, 4 = wheelchair bound.
 UL = upper limbs, LL = lower limbs; + = present; - = absent; NA = not available.

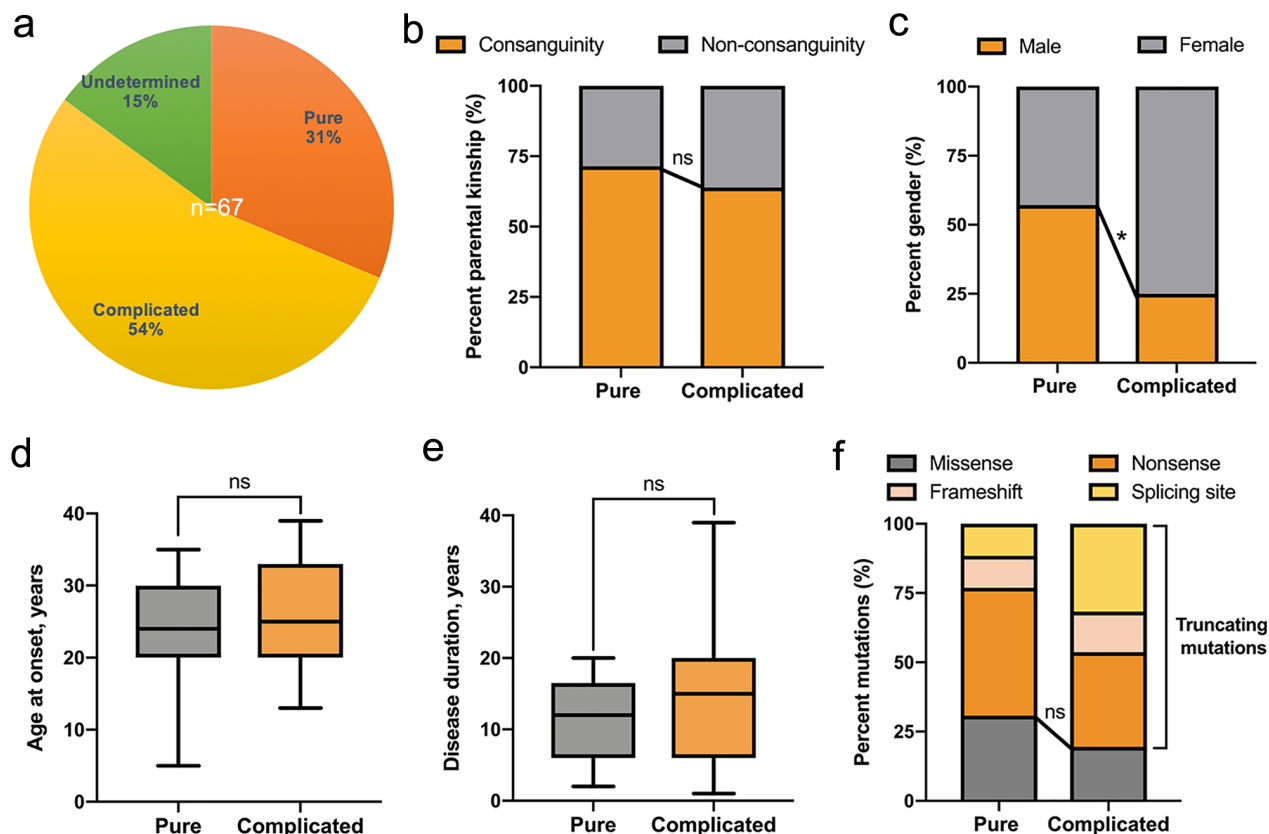


Figure 2. Comprehensive analysis of phenotypic heterogeneity for *CAPN1*-associated SPG76. (A) Distribution of pure, complicated, and undetermined forms (no available clinical information) across all reported SPG76 patients ($n = 67$). (B) Statistical comparison of the percentage of parental kinship between the pure and complicated forms of SPG76. Chi-squared followed by Bonferroni test. (C) Statistical comparison of the gender distribution between pure and complicated forms. Chi-squared followed by Bonferroni test. * indicates $P < 0.025$. (D) Statistical comparison of the age at onset between pure and complicated forms. Student's t -test. (E) Statistical comparison of the disease duration between pure and complicated forms. Student's t -test. (F) Statistical comparison of the percentage of truncating or missense mutation types between pure and complicated forms. Nonsense, frameshift, and splicing site mutations are combined into the truncating mutation category for comparison with missense mutations. In box and whisker plots, error bars indicate min to max. Chi-squared followed by Bonferroni test. ns, not significant.

exon-associated hotspot (Fig. 1B,C). Additionally, scrutiny of the integrated clinical information available for 85.1% (57/67) of the *CAPN1*-associated SPG76 patients showed that 36.8% (21/57) of these patients were pure form HSP (Fig. 2A). In contrast, 63.2% (36/57) of this patient subset presented as complicated HSP with cerebellar signs, and 25% (9/36) of which exhibited cerebellar atrophy.

To explore the factors (*i.e.*, gender, disease course, genetic background, etc.) potentially contributing to phenotypic heterogeneity, we first validated the parental relationship between pure form and complicated form HSP and found no significant differences in consanguinity (Fig. 2B). Subsequently, a comparison of gender distribution between the two groups indicated that female SPG76 patients were significantly more likely to present with

complicated HSP than male patients ($P = 0.015$, Fig. 2C). Given that slowly progressive paraparesis is the prototypical characteristic of HSP, we then evaluated age at onset and disease duration. However, this analysis revealed no significant differences in the disease course between pure form and complicated form HSP (Fig. 2D,E). We investigated the relationship between genotype and phenotype. No significant differences were found in the ratio of truncating mutations (*i.e.*, nonsense, frameshift, and splicing site mutations) versus missense mutations between the pure and complicated groups (Fig. 2F).

Discussion

In the current study, HTS analysis of 240 HSP families identified six additional autosomal-recessive HSP families,

with eight patients carrying six homozygous or compound heterozygous variants of *CAPN1*. Among them, the three homozygous variants (c.182_183insC (p.V64Gfs*103) in Families 1 and 4, c.759+1G>A in Family 3, and c.853C>T (p.R285*) in Family 5) were found to be known truncating mutations,^{6,11,21} while the three compound heterozygous variants ((c.1442G>A (p.R481Q) in Family 6, c.1493C>T (p.P498L) in Families 2 and 6, and c.1852C>T (p.R618W) in Family 2) were defined as novel, likely-pathogenic missense mutations. A combined total of 33 rare *CAPN1* mutations were detected between our cohort and previously reported cases.^{5,6,11-24} Notably, seven mutations (c.182_183insC, c.759+1G>A, c.853C>T, c.1015C>T, c.1142C>T, c.1176G>A, and c.1534C>T) were shared in ≥ 2 pedigrees, and in particular the nonsense mutation c.1176G>A (p.W392*) was most prevalent, recorded in four pedigrees.

In agreement with previous reports of HSP, the clinical features of the *CAPN1*-associated SPG76 patients also showed inter- and intra-family variability.^{5,15,18} Comprehensive analysis of all available clinical information for our cohort combined with that available in previous studies showed that lower limb spasticity, manifesting as stiffness, hyperreflexia, and Babinski signs, developed in about 94% of patients, followed by ataxia, dysarthria, and cerebellar atrophy.^{5,6,11-24} Meanwhile, the clinical overlap is extensive between complicated form HSP and HSP mimic diseases (e.g., adrenoleukodystrophy, spinocerebellar ataxia type 3, etc.).^{30,31} Remarkably, the variability and fluidity of the ataxia-spasticity disease spectrum suggest that spastic paraplegia and ataxia share not only overlapping phenotypes and causal genes, but also disease-specific underlying mechanisms (e.g., common cellular pathways or regulatory networks, etc.).⁹ This dilemma presents a highly challenging obstacle for correct clinical diagnoses of HSP. Moreover, an increasing suite of HSP-causative genes, including *PNPLA6* (SPG39), *KIF1C* (SPG58), *CAPN1* (SPG76), *ATP13A2* (SPG78), and *UCHL1* (SPG79), have been associated with hereditary ataxia.^{9,24}

To clarify the causes of phenotypic heterogeneity in *CAPN1*-associated SPG76 disease, we performed a comprehensive analysis of the parental kinship, gender, age at onset, disease duration, and mutation type between the pure and complicated HSP forms. Clinically, we speculated that mutations of *CAPN1* are more likely to lead to complicated HSP with cerebellar ataxia, especially for female SPG76 patients, rather than pure HSP. In addition, the consanguinity and disease course potentially do not substantially contribute to differences in clinical characteristics. Genetically, all mutations have been scattered along the conserved functional domains of calpain-1. Moreover, both truncating and missense mutations can

be accompanied by either pure or complicated forms of HSP. Thus, genotype does not ostensibly share a causative relationship with specific SPG76 phenotypes. However, on further review of the calpain-1 linker structure, we speculated that mutations in this location were more likely to cause pure form HSP than mutations in the above functional domains. Additionally, this heterogeneous SPG76 phenotypic spectrum could also rationally be accounted for by as-yet-unknown genetic modifiers or interacting proteins of *CAPN1*.^{27,32}

In conclusion, our study extends the mutation spectra and clinical profiles of *CAPN1*-associated SPG76 disease, stressing that *CAPN1* screening is warranted in both pure and complicated forms of HSP. We demonstrated that the factors of gender and calpain-1 linker structure may potentially contribute to SPG76 phenotype, although the extent of this contribution remains to be investigated. Further study of a large patient cohort and the use of patient-specific models (e.g., induced pluripotent stem cells),³³ will facilitate the elucidation of mechanisms underlying this characteristic phenotypic heterogeneity and variability for *CAPN1*-associated SPG76, as well as for other disease-causing genes in both as pure or ataxic HSP.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

X.L. and W.-J.C. carried out the study concept and design. X.L., L.-L.L., Y.-J.C., and Y.-L.L. wrote the initial manuscript and constructed the figures and tables. Y.-J.C., Y.-L.L., X.-H.L., M.-W.W., and E.-L.D. involved in acquisition of data and analysis. X.L., W.-J.C., and N.W. contributed to the critical revision of the manuscript, figures, and tables. All authors have read and approved the final manuscript.

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Supporting Information

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

Supplemental File S1. Tables for the primers of Sanger sequencing, and the information of novel mutations in *CAPN1*.

Supplemental File S2. Figures for the distribution of HSP in our cohort, and the Sanger tracing of mutations in *CAPN1*.