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Novel mutations in the *SPAST* gene cause hereditary spastic paraplegia

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ABSTRACT

Background: Mutations in the *SPAST* gene are the most frequent cause of hereditary spastic paraplegia (HSP). We aim to extend the mutation spectrum of spastic paraplegia 4 (SPG4) and carried out experiment in vitro to explore the influence of the *SPAST* gene mutation on the function of corresponding protein.

Methods: Whole-exome sequencing (WES) combined with multiplex ligation-dependent probe amplification (MLPA) were performed in a cohort of 150 patients clinically diagnosed with HSP. We focus on screening for mutations in *SPAST* gene and carrying out functional experiments to assess the effects of the novel variants.

Results: A total of 34 different mutations in the *SPAST* gene were identified, of which 10 were novel, including 1 missense (c.1479T > A), 1 nonsense (c.766G > T), 3 splicing (c.1413 + 1_1413 + 4delGTAA, c.1729-1G > A and c.1536 + 2T > G) and 5 frameshift mutations (c.1094delC, c.885dupA, c.517_518delAG, c.280delG and c.908dupC). For 7 novel non-splicing mutations, functional study showed that accumulated M1 spastin colocalized with microtubules which was different from a uniformly diffused M87 spastin. While an impairment in severing activity was observed in both mutant M1 and mutant M87, except for c.280delG. All 3 novel splicing variants w ere predicted to affect splicing by using bioinformatic programs. However, only c.1536 + 2T > G had no influence on splice site in vitro, which conflicts with the in-silico analysis.

Conclusion: We genetically diagnosed 40 SPG4 patients. All the novel non-splicing mutations except for c.280delG were certified to exert an effect on the microtubule-severing and all the novel splicing mutations other than c.1536 + 2T > G would cause abnormal splicing of the spastin.

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1. Introduction

Hereditary spastic paraplegia (HSP) is a highly genetically heterogeneous group of neurodegenerative disorders that manifest predominantly as progressive spasticity and weakness in lower limbs [1]. Currently, over 80 distinct genetic loci and more than 60 genes have been linked to HSP [2].

Spastic paraplegia type 4 (SPG4) caused by the *SPAST* gene mutation is the most common subtype, accounting for 40% and 20% of autosomal dominant and sporadic HSP cases, respectively [3]. In most cases, SPG4 is classified as a pure type of HSP with isolated pyramidal signs (spasticity, hyperreflexia and extensor plantar responses) [1]. More than 30% of SPG4 patients have sphincter disturbances or decreased vibration sensation at the ankles [4]. Manifestations accompanied by additional neurologic abnormalities, such as cognitive impairment, ataxia and seizures, which is considered a type of complicated HSP, have been reported [5,6].

SPAST has 2 main isoforms, named M1 (616 amino acids) and M87 (530 amino acids). M87 is the most abundant isoform in all tissues, while M1 is detectable only in the adult spinal cord, which is the location of axon degeneration in HSP patients [7]. Moreover, two other isoforms that lack 32 amino acids of either M1 or M87 might be generated due to alternative splicing of exon 4 [8].

Spastin, encoded by the *SPAST* gene, a member of the ATPase family, is related to the regulation of number, length and mobility of microtubules and is required for membrane trafficking [1,3,9–11]. Both the two main isoforms (M1 and M87) contain the microtubule-binding domain (MIT), microtubule interacting and trafficking domain (MTBD) and ATPase associated with various cellular activities (AAA) domains [1,8]. Domains in the N-terminus of spastin promote the targeting of spastin to specific sites. The MIT domain can be found in various endosomal proteins with roles in mediating interactions with charged multivesicular body protein (CHMP) members, which are related to membrane scission and is required for cytokinesis and membrane trafficking [1,12,13]. The HD domain, which is present only in the M1 spastin isoform, is predicted to form a hairpin that can partially insert into the lipid bilayer-like endoplasmic reticulum (ER) membrane and is involved in membrane curvature [8]. Domains in the C-terminus of spastin containing MTBD and AAA are related to microtubule severing. The MTBD domain is required for spastin to bind microtubules in an ATP-independent manner before severing, and the AAA domain can catalyze microtubule breakage [1,14].

Here, we analyzed the clinical characteristics and genetic features of 40 patients with SPG4 molecular diagnoses using WES combined with Sanger sequencing and MLPA and carried out functional studies for the novel variants we identified in the SPG4 patients.

This study is part of a larger study aimed at genotype-phenotype analysis of HSP patients in China.

2. Methods

2.1. Participants

The samples are a subset of our genotype-phenotype analysis study consisting of 150 HSP patients and 1500 controls in China. Informed consents were signed by all participants. This study was approved by the ethics committees of Ruijin Hospital affiliated with Shanghai Jiao Tong University School of Medicine.

2.2. Whole-exome sequencing and data analysis

Genomic DNA was extracted by the phenol/chloroform method. Whole-exome sequencing was carried out for 150 probands using Agilent SureSelect v6 reagents and Illumina HiSeq X Ten platform for exome capture and sequencing. All common variants for which the minor allele frequency (MAF) was higher than 1% based on public databases of human variation (1000 Genomes Project, ExAC and gnomAD) were eliminated. We screened variants of known pathogenic genes of HSP and focus on variants in *SPAST* gene. Further interpretations were carried out according to the American College of Medical Genetics and Genomics (ACMG) guidelines. Sanger sequencing was applied to validate the potential pathogenic variants.

2.3. Multiplex ligation-dependent probe amplification assay

Rearrangement mutations constitute approximately 20%–25% of *SPAST* pathogenic variants [4]. We carried out an MLPA assay for all patients with negative WES analysis using SALSA P165–C2 (MRC-Holland, the Netherlands) for the MLPA reaction, following fragment separation by capillary electrophoresis. The analysis of MLPA data was based on MLPA DNA Protocol version MDP-005, which is available at http://www.mlpa.com.

2.4. Preparation of spastin constructs

Plasmid constructs were divided into three groups, designated Groups I, II and III. Human cDNA encoding full-length spastin (amino acids 1–616) with 221 nucleotides of the 5′ UTR was purchased from Genecreate Technology (Wuhan, China) for Group I constructs which were predicted to express the two main isoforms simultaneously. To generate wild-type (WT) constructs for Group II and Group III that expressed only M1 and M87 spastin isoforms separately, cDNA with a good Kozak's sequence (accATGa) substituting for the Kozak's sequence (tgaATGa) surrounding the first start codon and cDNA lacking the first 86 amino acids with a replacement of the Kozak's sequence (ctcATGg) surrounding the M87 start codon with accATGg were prepared, both of which were amplified from the WT construct of Group I by polymerase chain reaction (PCR) using specific primers. Mutations c.280delG, c.517_518del, c.766G > T, c.885dupA, c.908dupC, c.1094delC, c.1479T > A and c.1343G > A were introduced into the WT constructs by site-directed mutagenesis, as well as the mutation c.1343G > A, which has been proven to affect the function of spastin [15]. The reference sequence NM_014946 was used for assigning the mutations. All target fragments were cloned into the pcDNA3.1 vector.

2.5. Cell culture and transfections

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (HyClone, USA) containing 10% fetal bovine serum (Gibco, USA). Cells were transfected with spastin constructs, empty plasmids and minigenes using Lipofectamine 3000 (Invitrogen, USA).

2.6. Immunoblotting of cultured cells

Thirty-six hours after transfection, protein was extracted using RIPA Lysis Buffer (Beyotime, China) with proteinase inhibitor (10% cocktail and 1% PMSF), separated by SDS-PAGE electrophoresis and immunoblotted with an anti-spastin (A2) mouse monoclonal antibody (1:1000, Santa Cruz, USA) or anti beta-actin mouse monoclonal antibody (1:2500, Sigma-Aldrich, USA) and horseradish peroxidase (HRP) - conjugated goat anti-mouse second antibody (1:10,000, Invitrogen, USA).

2.7. Immunostaining of cultured cells

Thirty-six hours after transfection, cells were fixed with 4% paraformaldehyde and postextracted with 0.1% Triton X-100. Subsequently, cells were incubated with an anti-spastin (A2) mouse monoclonal antibody (1:200, Santa Cruz, USA) overnight at 4 °C, followed by exposure to Alexa Fluor-488-conjugated goat anti-mouse (1:1000, Invitrogen, USA) and Alexa Fluor-594-conjugated rabbit anti-beta-tubulin monoclonal antibodies (dilution 1:200, Cell Signaling Technology, USA) for

1 h at 37 °C. Images were obtained with a Zeiss 710 confocal microscope.

Minigene Reporter Assay.

Bioinformatic Predictions of Splicing Alterations.

BDGP, NetGene2 and HSF were used to evaluate the effects of variants on splice sites.

2.8. Preparation of minigenes

The *SPAST* exons relevant to 3 novel splicing variants (c.1413 + 1_1413 + 4delGTAA, c.1729-1G > A and c.1536 + 2T > G) along with flanking intronic sequences were amplified by PCR from patients P1141, P2193 and P3390 with gDNA. The PCR products of c.1413 + 1_1413 + 4delGTAA and c.1729-1G > A were cloned into the pcDNA3.1 vector, whereas the products of c.1536 + 2T > G were inserted into the pcMINI vector.

2.9. Functional splicing analysis

Minigenes were transfected into HeLa cells. The RNAiso Plus kit (Takara, Japan) was used for total RNA extraction, followed by RT-PCR using the PrimeScript RT reagent kit (Takara, Japan) to generate related cDNA. Electrophoresis on 2% agarose gels and Sanger sequencing were used for splicing pattern analysis.

3. Results

3.1. Clinical manifestations

77 sporadic patients and 73 probands with family history were included in our cohort. Of these, 16 sporadic patients and 24 familial patients in autosomal dominant inheritance pattern carried *SPAST* mutations. The average age of onset was 30.9 ± 14.8 years with a disease duration of 15.5 ± 7.6 years (range 3–35). 8 patients need assistance within the disease progress and none of them are wheelchair-bound. The average age when patients need walking-assistance is 12.63 ± 5.13 years. Gender had no significant influence on age of initial onset or the course of the disease. All patients in our cohort were manifested as pure HSP. A detailed description of the clinical manifestations is shown in Table 1.

3.2. Genetic findings

A total of 34 mutations in the *SPAST* gene were detected, which were classified as pathogenic or likely pathogenic variants according to the ACMG guidelines [16] (Table 1). Among the 40 SPG4 patients, 31 carried sequence variants (77.50%), and 9 were found to have rearrangement variants (22.50%). 3 missense mutations (c.1841C > T, c.1495C > T and c.1396C > G), 7 nonsense mutations (c.1573C > T, c.1555 A > T, c.508C > T, c.838C > T, c.1291C > T, c.734C > G and c. 430C > T), 5 splicing mutations (c.1728 + 1G > C, c.1728 + 1G > A, c.1245 + 1G > A, c.1536 + 1G > T, c.1494-2delA), 2 deletion mutations (c.458delT and c.1035delT) and all 7 (multi)exon deletion or duplication variants have been reported or listed in the HGMD Professional or clinvar database, whereas 10 mutations were novel, including 1 missense mutation (c.1479T > A), 1 nonsense mutation (c.766G > T), 3 splicing mutations (c.1413 + 1_1413 + 4delGTAA, c.1729-1G > A and c.1536 + 2T > G) and 5 frameshift mutations (c.1094delC, c.885dupA, c.517_518delAG, c.280delG and c.908dupC) (Fig. 1) [17]. All the novel mutations were absent in the 1500 normal controls. The detailed bioinformatic data are shown in Table 1.

3.3. Functional study

Severing Activity of Mutants M1 and M87 Was Decreased, and Only Mutant M1 Accumulated Abnormally.

M1 and M87 were expressed simultaneously in cells transfected with Group I wild-type constructs, as well as N296K, T305Y, P365L and D493E (Fig. S1). We further explored the impacts of the mutant M1 and M87 isoforms on the microtubule-severing activity of spastin. Group II and III spastin constructs were transfected into HeLa cells. All the isoforms in group II were detected except for A95P, and all the M87 isoforms in group III were detected apart from A95P, R173S and G256X (Fig. S1). Spastin distributed evenly or formed clumps in the cytoplasm, which were sufficient to sever all microtubules in wild type of both M1 and M87 (Fig. 2). A reduction in severing activity was shown in all the mutation groups of M1 and M87, apart from A95P, in which mutant spastin isoforms were not detected. Furthermore, mutant M1 spastin isoforms were colocalized with microtubules and accumulated around the nucleus, while mutant M87 spastin was uniformly diffuse in the cytoplasm of transfected cells (Fig. 2).

3.4. Splicing mutations assessment

Two variants (c.1413 + 1_1413 + 4delGTAA and c.1536 + 2T > G) were predicted by all algorithms to seriously affect splicing. The third variant, c.1729-1G > A, located at position −1 of exon 18, was predicted to modify the splice site by HSF, although the canonical splice sites of exon 18 were not predicted by BDGP and NetGene2.

3.5. Analysis of the variant c.1413 + 1_1413 + 4delGTAA

A minigene comprising exons 10, 11 and 12 of the *SPAST* gene was constructed and transfected into HeLa cells (Fig. 3A). Three different RT-PCR fragments were found in wide-type group, whereas only two fragments were shown in cells transfected with the mutant minigene (Fig. 3B).

The longest fragment appeared only in the wild type (WT1), while the medium- and short-length products could be found both in the WT1 and mutant groups (Mut1). Sanger sequencing of RT-PCR products demonstrated that the longest fragment comprised exons 10, 11 and 12, the medium-length fragment in WT1 and Mut1 completely skipped exon 11, and the short fragment in WT1 contained exon 10 followed by an indistinguishable sequence as well as that in Mut1 (Fig. 3C). In conclusion, the wild-type minigene had three alternative splicing variants; in contrast, the mutated minigene had only two alternative splicing variants that could not produce a mature mRNA with exon 11.

3.6. Analysis of the variant c.1536 + 2T > G

We constructed a minigene comprising *SPAST* exon 13, exon A and exon B (Fig. 3D). The length of RT-PCR products in the wild-type group (WT2) was not significantly different from that in the mutant group (Mut2) (Fig. 3E). Furthermore, Sanger sequencing revealed that the mRNAs produced by both the WT2 and Mut2 minigenes comprised exons A, B and 13. Variant c.1536 + 2T > G did not influence the splice site (Fig. 3F).

3.7. Analysis of the variant c.1729-1G > A

Wild-type (WT3) and mutant minigenes containing exons 16 and 17 (Mut3) were constructed (Fig. 3G). Agarose gel electrophoresis revealed that the RT-PCR fragment obtained in Mut3 was smaller than that obtained in WT3 (Fig. 3H). Direct sequencing of RT-PCR products showed that the mutant minigene produced mRNAs with an incomplete exon 17(Fig. 3I).

4. Discussion

We genetically analyzed 73 familial and 77 sporadic HSP patients in China and found that 24 AD-HSP and 16 of sporadic patients harbor

Table 1
Clinical and genetic features of 40 patients.

Patient	Sex	AAO (years)	DD (years)	Inher-itance	Variant		ACMG	Hypertonia		Hyperreflexia		Weakness		Ankleclonus	Extensor plantar responses	Additional features	Walking-assistance (years)
					cDNA	Protein		UL	LL	UL	LL	UL	LL				
P0108	M	14	27	AD	Exon 1 del	NA	P	–	+	+	+	–	–	–	+	–	N
P0283	M	50	14	AD	c.766G > T *	p.G256X	P	–	+	–	+	–	+	+	+	SG	Y (5)
P0284	F	30	32	AD	c.1728+1G > C	NA	P	–	+	–	+	–	+	+	–	SG	Y (23)
P0520	M	48	16	AD	c.1728+1G > A	NA	P	–	+	–	+	–	–	–	+	SG	N
P0642	F	20	12	AD	c.1094delC *	p.P365Lfs	P	–	+	–	+	–	–	+	+	SG	N
P0862	M	18	20	AD	c.1573C > T	p.Q515X	P	–	+	–	+	–	–	+	+	–	N
P0942	F	2	14	AD	c.1728+1G > A	NA	P	–	–	–	–	–	–	–	+	–	N
P0966	M	32	13	S	c.1841C > T	p.T614I	LP	–	+	–	+	–	–	+	–	SG	N
P1051	M	20	23	AD	c.458delT	p.I153Mfs	P	–	+	–	+	–	–	+	+	SG	N
P1068	M	36	20	AD	c.458delT	p.I153Mfs	P	–	+	–	–	–	–	–	–	SG	Y (10)
P1089	M	42	18	S	c.1245+1G > A	NA	P	–	+	–	+	–	+	–	+	SG, PC	N
P1113	M	18	14	AD	c.1291C > T	NA	P	–	+	–	+	–	+	+	+	–	Y (10)
P1141	M	50	9	S	c.1413 + 1_1413+4delGTAA *	NA	P	–	+	–	+	–	+	+	+	SG	N
P1227	F	26	19	AD	c.1555A > T	p.L519X	P	–	+	–	+	–	+	–	+	SG	Y (14)
P1267	M	32	35	AD	c.885dupA *	p.N296Kfs	P	–	+	+	+	–	+	+	+	SG	N
P1276	F	44	18	S	c.517_518delAG *	p.R173Sfs	P	–	+	+	+	–	+	–	+	–	N
P1339	M	34	9	S	c.280delG *	p.A95Pfs	P	–	–	–	+	–	+	+	+	SG	N
P1357	M	46	8	AD	c.508C > T	p.Q170X	P	–	+	–	+	–	+	+	+	SG	N
P1481	M	37	17	AD	c.1035delT	p.G346Vfs	P	–	+	–	+	–	+	+	–	PC	N
P1702	F	66	13	AD	Exon 1 dup	NA	P	–	+	–	+	–	+	–	+	PC	Y (12)
P1872	M	40	17	AD	Exon 1–4 del	NA	P	–	+	–	+	–	+	+	+	–	Y (14)
P1965	M	46	13	S	c.838C > T	p.Q280X	P	+	+	+	+	–	+	+	–	SG	N
P2108	M	22	9	S	c.1479T > A *	p.D493E	LP	–	+	–	+	–	+	+	–	SG	N
P2138	F	4	35	AD	Exon 5–7 del	NA	P	–	+	+	+	–	+	+	–	SG	N
P2193	M	40	10	S	c.1729-1G > A *	NA	P	–	+	–	+	–	+	+	+	–	N
P2450	M	11	21	S	c.1291C > T	p.R431X	P	–	+	–	+	–	+	+	+	SG	N
P2610	M	45	7	S	c.908dupC *	p.T305Yfs	P	–	+	–	+	–	+	+	+	SG	N
P2616	M	7	21	AD	c.1536+1G > T	NA	P	–	+	+	+	–	+	–	+	SG	N
P2618	M	29	15	S	c.1495C > T	p.R499C	LP	–	+	+	+	–	+	+	+	SG	Y (13)
P2958	F	0	3	AD	c.1536+1G > T	NA	P	+	+	+	+	–	–	+	–	–	N
P3390	M	32	14	S	c.1536+2T > G *	NA	P	–	+	–	+	–	+	+	+	SG	N
Z0323	M	30	21	AD	Exon 13–15 del	NA	P	–	+	–	+	–	+	–	+	–	NA
Z0441	F	43	10	S	Exon 6 del	NA	P	+	+	+	+	–	–	+	+	SG	N
Z0456	M	34	4	S	c.1396C > G	p.L466V	LP	–	+	+	+	–	+	–	–	–	N
Z0952	F	42	11	S	c.734C > G	p.S245X	P	–	+	–	+	–	–	+	–	–	NA
Z1004	M	25	14	AD	Exon 2–17 del	NA	P	–	+	–	+	–	–	–	–	–	NA
Z1014	M	25	19	S	c.430C > T	p.Q144X	P	–	+	–	+	–	–	+	+	–	NA
Z1535	M	20	9	AD	Exon 5–7 del	NA	P	–	+	–	+	–	–	+	+	–	N
Z1588	M	42	8	AD	Exon 6 del	NA	P	–	+	–	+	–	–	–	+	–	N
X0002	M	34	7	AD	c.1494-2delA	NA	P	–	+	+	+	–	–	+	+	SG	N

M = male; F = female; AD = autosomal dominant inheritance; S = sporadic; P = pathogenic; LP = likely pathogenic; UL = upper limbs; LL = lower limbs; SG = scissors gait; PC = pes cavus; Y = yes; N = no; NA = not available.

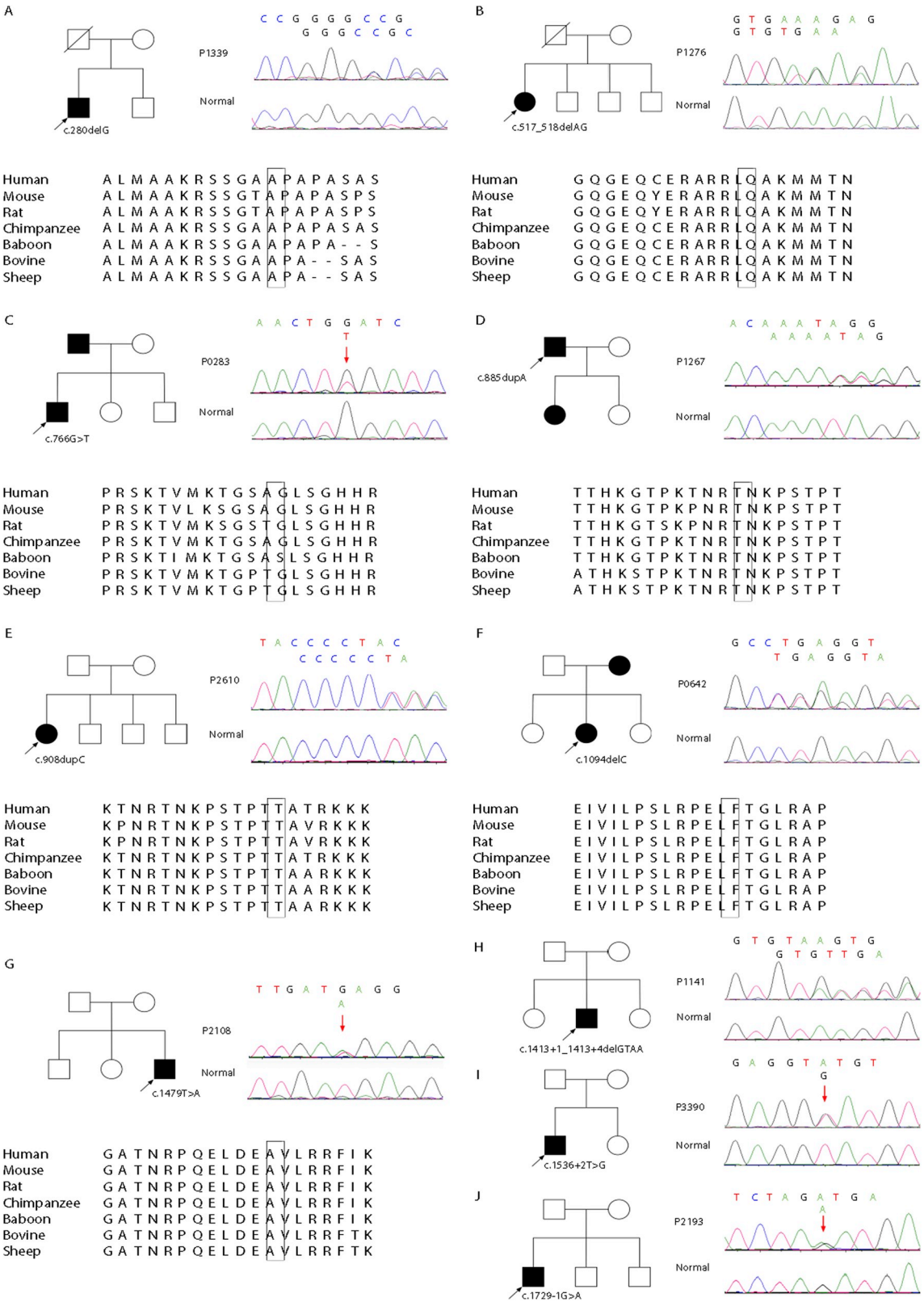

Fig. 1. Novel *SPAST* mutations identified in our cohort.

SPAST causative mutations. All SPG4 patients, including 30 males and 10 females, had the pure form of HSP. The age (30.9 ± 14.8 years) and gender manifestations were the same as those reported in the neuromuscular disease database (https://neuromuscular.wustl.edu). To date, nearly 700 mutations in the *SPAST* gene, including 542 subtle mutations and 141 rearrangements, have been reported according to the HGMD Professional database. In our cohort, 77.50% (n = 31) carried subtle mutations and 22.50% (n = 9) had exonic rearrangements, consistent with previous research [4]. Compared with rearrangements, point mutations showed a similar phenotype but an earlier age of onset

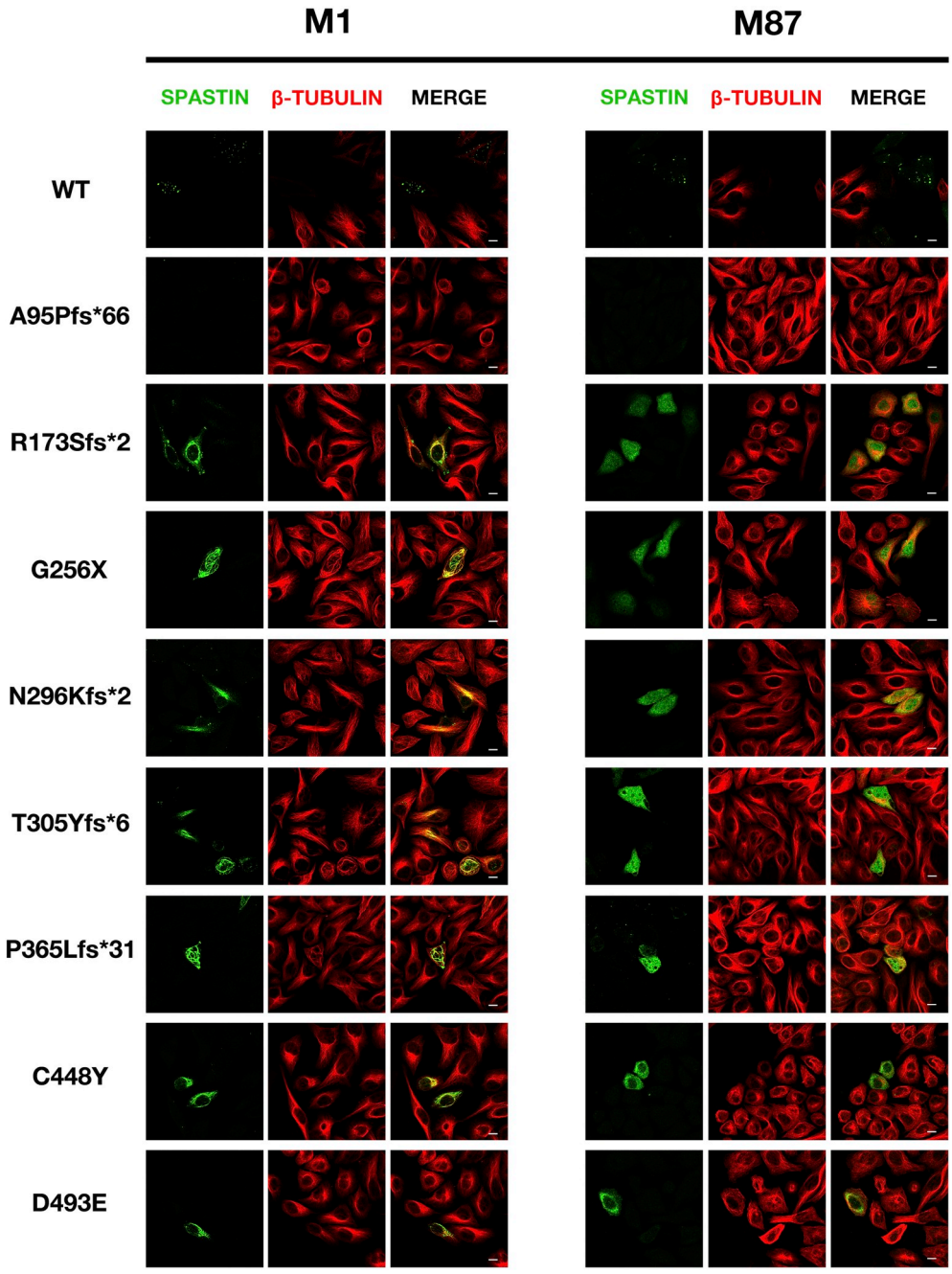

Fig. 2. The severing activity of spastin was affected in all the mutation groups of M1 and M87 except for A95P. Mutant M1 spastin isoforms were colocalized with microtubules and accumulated around the nucleus, while mutant M87 spastin diffused uniformly in the cytoplasm of transfected cells. (Bar = 10 μm).

[18]. However, no significant difference was shown in the age of onset between patients with rearrangements and subtle mutations in our study.

Initial codons are recognized more efficiently with a good context g (a)ccATGg [1,19,20]. Around the first initial codon of *SPAST*, the Kozak sequence (tgaATGa) deviates considerably from the consensus sequence, whereas a better Kozak sequence ctcATGg is present surrounding the M87 initial codon. In addition, a good Kozak sequence (gttATGg) present in an upstream ORF that partially overlaps with the main *SPAST* ORF may also inhibit translation of the initial codon [20]. As shown in Fig. S1, the constructs in Group I contained full-length spastin and the 5' UTR expressed M1 and M87 isoforms simultaneously at different levels. M1-A95P, M87-A95P, M87-R173S and M87-G256X were not detected.

All the missense and splicing mutations in our study presented in the AAA domain, whereas frameshift and nonsense mutations could be found in other domains of spastin. To sever microtubules, the AAA domain of spastin assembles into hexamers, docks on the microtubule lattice and tugs the C-terminal tail of tubulin through the central pore of the hexamer using ATP hydrolysis [1,21,22]. Missense mutation D493E, which is located in the AAA domain, was predicted to have adverse effects on protein function. Otherwise, all the novel frameshift and nonsense mutations would lead to premature termination, which was also detrimental to the translation of the AAA domain of functional spastin. As expected, microtubules were severed in the WT group but not in cells transfected with mutant spastin, which was similar to the pathogenic mutation C448Y (Fig. 2) [15]. Both M1-and M87-A95P were not shown. The targeted amino acids of the anti-spastin (A2) mouse

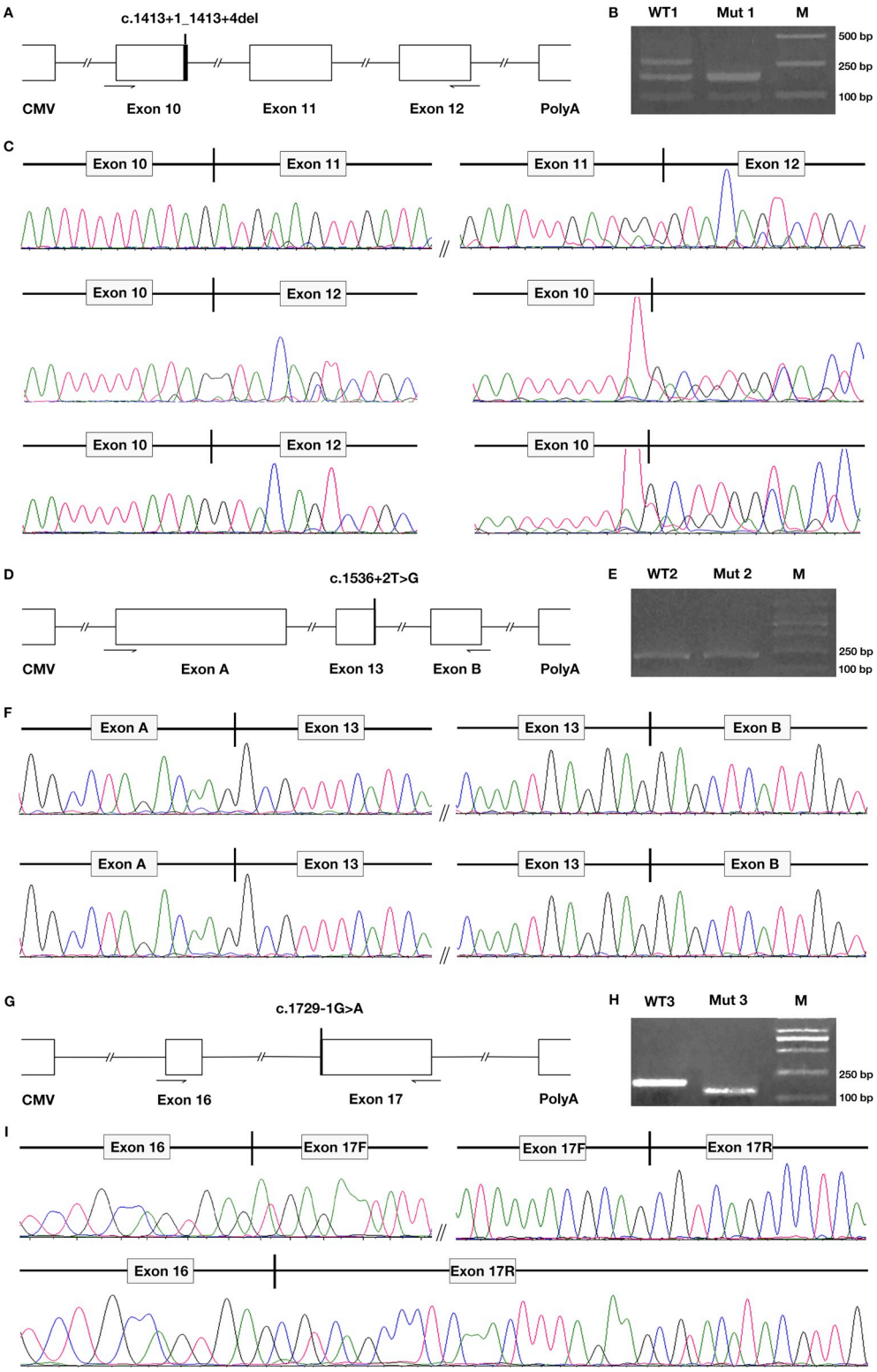

Fig. 3. Minigene splicing experiment for three novel splicing mutations. Schematic diagrams of the recombinant expression vectors with variants c.1413 + 1_1413 + 4 del GTAA, c.1536 + 2 T > G and c.1729-1 G > A are shown in A, D and G. Agarose gel electrophoresis of RT-PCR fragments are shown in B, E and H. The Sanger sequences of the fragments are shown in C, F and I.

monoclonal antibody mapped near the N-terminus of spastin (61–194). The premature termination of spastin with mutation A95P might influence the antibody-antigen interaction.

The severing activity of spastin breaks long microtubules and creates a mass of free microtubule ends that is critical for microtubule transport, axonal growth and axonal branch formation. Furthermore, most pathogenetic mutations in the *SPAST* gene have deleterious effects on the microtubule binding, spastin assembly or ATP hydrolysis required for microtubule severing [1]. Haploinsufficiency has become the most commonly postulated explanation for SPG4 [1,23]. However, there remain concerns about the loss of function (LOF) model: (1) degeneration occurs mostly in the corticospinal tracts; (2) according to the

Neuromuscular database (https://neuromuscular.wustl.edu), SPG4 is typically adult onset (3rd to 5th decades) and no developmental abnormalities appear in SPG4 patients; (3) no correlation was revealed between the severity of symptoms and the level of spastin; and (4) several mutations that have no influence on severing activity can also cause SPG4 [15,24,25]. In addition, the toxicity of mutated spastin was tested. Several studies have revealed that mutated M1 is most relevant to the cytotoxicity of SPG4 [24,26,27]. Additionally, M1 was detectable only in the adult spinal cord, which was more relevant to the degeneration of corticospinal tracts than M87 [15]. Haploinsufficiency may aggravate the cellular defects induced by the toxicity of mutant spastin through a gain-of-function (GOF) mechanism [24]. Our present studies on HELA cells, consistent with previous research, demonstrate that mutant M1, rather than mutant M87, decorated a subpopulation of microtubules (Fig. 2). Novel variants in coding sequence might be harmful through both GOF and haploinsufficiency mechanisms. For cells transfected with A95P spastin, neither mutant M1 nor M87 was detected by immunofluorescence. Although the level of microtubules was higher than that of the WT, the functional change of mutant spastin-A95P was still unknown.

For the three novel splicing variants, we used BDGP, NetGene2 and HSF to evaluate the influence on splicing. All variants were predicted to influence the splice site. However, only variants c.1413 + 1_1413 + 4delGTAA and c.1729-1G > A affect splicing by experimental verification, and the influence of c.1536 + 2T > G is still unknown. Although several bioinformatic programs exist to predict effects on splice sites, minigene reporter assays are important for diagnosis.

Overall, our study identified 40 SPG4 patients and analyzed their clinical and genetic characteristics. We further proved the influence of the novel mutation on the function of spastin by in vitro experiments. These findings extend the mutation spectrum of SPG4, which is valuable for diagnosis of the disease.

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Author contributions

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Dr. Zhao: data acquisition, analysis and interpretation of data, statistical analysis.

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Prof. Cao: funding, study design and conceptualization, data acquisition, analysis and interpretation of data, statistical analysis, manuscript revision.

Declaration of competing interest

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Appendix A. Supplementary data

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