Original Article

The emerging genetic diversity of hereditary spastic paraplegia in Korean patients

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ABSTRACT

Hereditary Spastic Paraplegias (HSP) are a group of rare inherited neurological disorders characterized by progressive loss of corticospinal motor-tract function. Numerous patients with HSP remain undiagnosed despite screening for known genetic causes of HSP. Therefore, identification of novel genetic variations related to HSP is needed. In this study, we identified 88 genetic variants in 54 genes from whole-exome data of 82 clinically well-defined Korean HSP families. Fifty-six percent were known HSP genes, and 44% were composed of putative candidate HSP genes involved in the HSPome and originally reported neuron-related genes, not previously diagnosed in HSP patients. Their inheritance modes were 39, *de novo*; 33, autosomal dominant; and 10, autosomal recessive. Notably, *ALDH18A1* showed the second highest frequency. Fourteen known HSP genes were firstly reported in Koreans, with some of their variants being predictive of HSP-causing protein malfunction. *SPAST* and *REEP1* mutants with unknown function induced neurite abnormality. Further, 54 HSP-related genes were closely linked to the HSP progression-related network. Additionally, the genetic spectrum and variation of known HSP genes differed across ethnic groups. These results expand the genetic spectrum for HSP and may contribute to the accurate diagnosis and treatment for rare HSP.

1. Introduction

Hereditary spastic paraplegias (HSP) are a clinically and genetically heterogeneous group of rare neurodegenerative disorders. Pure HSPs are characterized by progressive spasticity and weakness, extensor plantar responses, and hyperreflexia of deep tendon reflexes in the lower limbs.

Complicated HSPs are characterized by additional neurological or non-neurological features [1,2]. Pure HSPs are more prevalent than complicated HSPs; moreover, patients with pure HSPs mostly have normal lifespans [3]. Furthermore, HSPs have been identified as autosomal dominant (AD), autosomal recessive (AR), X-linked, and maternally mitochondrial-inherited forms; moreover, 75–80% of affected

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individuals have AD-HSP [4].

Currently, > 80 genetic subtypes of HSP have been reported [4–7]. Spastic paraplegia 4 (SPG4), caused due to mutations in *SPAST*, is the most common type that accounts for 40% of AD-HSP cases [4,7]. SPG3A, which has a frequency of 10–15%, and disease-causing variants in *ATL1* are the main causes of early-onset AD-HSP. SPG30 in *KIF1A* and SPG31 in *REEP1* each account for 5% of AD-HSP cases. Other AD-HSP types with a predominantly adult onset are relatively rare and sporadic. Although previous studies have yielded promising findings, there remain cases with unidentified causative genes [8].

There have been large-scale studies and interpretation trials on HSP in several ethnic groups, including Chinese and Italians [5,9], with advances in sequencing techniques. These studies have reported varying HSP prevalence rates across several populations [10]; further, there were high levels of genetic divergence [11]. However, a global perspective of HSP cannot sufficiently facilitate gene discovery and a full mechanistic understanding of the disease. Specifically, studies on Korean patients with HSP have mainly focused on genetic variations within a few spastic paraplegia genes (SPG), including *SPAST*, *ATL1*, *SPG11*, and *REEP1*[12–15]. To obtain genome-wide genotype data on HSP disease in Korean patients, this study aimed to analyze whole-exome sequencing (WES) data obtained from 166 samples from 132 Korean patients with HSP and to determine the HSP-related genetic variation profile, which could demonstrate the genetic diversity among Korean patients with HSP. By comparing with data obtained from five ethnic groups, we explored the heterogeneous genetic spectrum and variations of HSP-causative genes. This study could provide clues for the development of novel diagnosis and therapeutic approaches for HSP.

2. Materials and methods

2.1. Human participants

We collected 166 samples from 104 unrelated Korean families. Patients who met the clinical diagnostic criteria for HSP were included regardless of their genetic diagnoses [1]. Each patient underwent a detailed neurological examination conducted by a board-certified neurologist at Samsung Medical Center (SMC). Major clinical assessments were performed, including assessment of demographic information, family history, developmental history, Spastic Paraplegia Rating Scale (SPRS), and HSP primary symptoms (lower extremity weakness/ spasticity/sensory impairment/bladder dysfunction). Moreover, we assessed additional symptoms, including ataxia, optic atrophy, optic neuropathy, pes cavus, peripheral neuropathy, and others. Written informed consent was obtained from all participants. Ethical approval for this study was obtained from the Institutional Review Board and Ethics Committee at the SMC and Korea Research Institute of Bioscience and Biotechnology.

2.2. Direct sequencing

Initially, 18 individuals underwent genomic DNA extraction from peripheral blood leukocytes using a Wizard Genomic DNA Purification kit, following the manufacturer's instructions (Promega Corporation, Madison, Wisconsin). All coding exons and flanking introns of the *ATL1* and *SPAST* genes were amplified through polymerase chain reaction using self-designed primers. Sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction kit, version 2.0 (Applied Biosystems, Foster City, California) on the ABI Prism 3100 genetic analyzer (Applied Biosystems).

2.3. Whole-exome sequencing (WES) and variation analysis

For the remaining 149 individuals, we prepared sequencing libraries from primary DNA extracted from leukocytes in blood samples using the TruSeq library preparation kit (Illumina, San Diego, CA, USA), following

the manufacturer's instructions. The Nextera Rapid Capture Exome kit (Illumina) was used to selectively amplify the coding regions of the genome, following the manufacturer's instructions. For accurate analysis, we performed 100× high-throughput paired-end WES using the Illumina HiSeq 2500 sequencer. Sequencing data were mapped to the GRCh38/hg38 human reference genome using Burrows-Wheeler Aligner software (v0.7.17) [16]. Preprocessing and identification of single-nucleotide variants and insertions-deletions were performed using the best practice protocol of the Genome Analysis Toolkit (GATK v4.1.5.0, Broad Institute, MA, USA) [17] and Pindel [18]. The ANNOVAR software (Version 2018Apr16) was used for functional annotation of all variants; moreover, we selected variants in HSP-related genes as candidates [19]. To determine whether the identified variants were novel or known, we compared them using the Single Nucleotide Polymorphism Database (dbSNP) (https://ncbi.nlm.nih.gov/snp) and previous reports. Finally, all variants were validated through Sanger sequencing using the ABI3730 DNA sequencer and multiplex ligation-dependent probe amplification.

2.4. Variation prioritization and pathogenicity estimation

In preliminary filtering, we removed variants with a minor allele frequency > 2% using the dbSNP, 1000 Genomes Project (https://www.internationalgenome.org/), and Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/). We selected variants in known and putative candidate HSP genes from the HSPome network in previous studies [20], as well as neuronal-related genes, and excluded variants present in unaffected individuals. The resulting variants were assigned as a kHSP (Korean HSP) gene set. Furthermore, benign or likely benign variants were removed using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and Clinical Genome Resource (ClinGen, https://clinicalgenome.org/). We used the American College of Medical Genetics (ACMG) classification for pathogenicity evaluation [21]. To predict the pathogenic effects of variants of uncertain significance (VUS) following the ACMG classification, we applied five algorithms in ANNOVAR, including the Sorting Intolerant from Tolerant, Polymorphism Phenotyping v2, MutationTaster, Likelihood Ratio Test, and Functional Analysis through Hidden Markov Models algorithms. Variants were considered as damaging when ≥ 3 of the 5 algorithms were satisfied.

2.5. Ethnic-based comparison of five representative HSP genes

We collected HSP gene sets from previous HSP studies on four ethnic groups for comparison with our data [5,9,22,23]. These groups comprised the Chinese (CN), French Canadian (FC), Hungarian (HU), and Italian (IT) groups based on ethnic information described in each study. Chinese datasets were collected from 41 reported studies in China. Each group comprised its HSP cohorts, with various analysis methods being used to explore HSP-related genes. These groups are representative given that they comprised the largest reported datasets in each of the countries. Although there were large-scale datasets from other journals, they lacked clear clinical or ethnic information. We collected their disclosed information indicative of the significance of their results. The gene frequency and mutation type were compared to confirm the composition differences across the ethnic groups. Five genes, including *SPAST*, *ATL1*, *SPG11*, *SPG7*, and *CYP7B1*, were selected to compare distribution among ethnic groups. These five genes were shared in at least three ethnic cohorts and were the most frequent in each cohort. Moreover, they are representative genes known to cause HSP [4].

2.6. Ontology and network analysis with HSP candidate genes

We investigated the phenotype ontology (PO) and genotype ontology (GO) distribution of the kHSP gene set using the GSEApy (https://pypi.

org/project/gseapy) with a significant *p*-value set at < 0.05. We analyzed the relationships among kHSP genes using IPA software (http://www.ingenuity.com) to explore the interactions between known and candidate HSP genes. We performed analysis to determine direct and indirect relationships of the variant effects. To establish a set of potential target candidate HSP genes, we collected all genes located on a network after excluding genes not linked with known or putative candidate HSP genes. Finally, we comprehensively integrated all molecular and cellular functions, physiological system development and function, and the related network functions based on the phenotypic features of HSP.

2.7. Protein modeling prediction

The three-dimensional structure was modeled using the available protein sequence by the web-based server Phyre2 [24] and analyzed using PyMol (The PyMOL Molecular Graphics System; http://www.pymol.Org).

2.8. Mutational function study on SPAST and REEP1

2.8.1. Antibodies and reagents

We purchased anti-α-tubulin, anti-β-actin, and anti- NF-L from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-acetylated α-tubulin and MAP2 were purchased from Cell Signaling Technology (Beverly, MA, USA). We obtained retinoic acid and DAPI solution from Sigma-Aldrich (St. Louis, MO, USA).

2.8.2. Cell culture and differentiation

We maintained HEK293 cells (American Type Culture Collection, Manassas, USA) in Dulbecco's modified Eagle medium (DMEM)/high glucose with L-glutamine and sodium pyruvate (HyClone, Logan, UT, USA), which was supplemented using 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Gaithersburg, MD, USA). We maintained human SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, USA) in DMEM/high glucose with sodium pyruvate without L-glutamine (HyClone, Logan, UT, USA), which was supplemented using 1% non-essential amino acid, 1% GlutaMAX, 10% FBS, and 1% penicillin/streptomycin (Gibco, Gaithersburg, MD, USA). The cells were maintained in a 5% CO_2 incubator at 37 °C. Regarding the neuronal differentiation conditions, SH-SY5Y cells were treated using retinoic acid (10 μM; Sigma-Aldrich, St. Louis, MO, USA) for 72 h. HEK293 and SH-SY5Y were transfected using the Lipofectamine 2000.

2.8.3. Plasmid DNA construction

The plasmid of human wild-type *SPAST* cloned into the pEGFP-C2 vector was previously described [25], and the human wild-type *REEP1* vector was purchased from OriGENE (Rockville, MD, USA; RC228141). The *SPAST*-mutant (S399W, T486I, F404del, and L397_L398del) vectors and *REEP1*-D116N vector were introduced using the GeneArt Site-Directed Mutagenesis PLUS system (Invitrogen, Waltham, MA, USA), as per the manufacturer's instructions. The *SPAST*-S399W, *SPAST*-T486I, *SPAST*-F404del, and *SPAST*-L397_L398del plasmids were subcloned into the pEGFP-C2 vector, while *REEP1*-wild type and *REEP1*-D116N were subcloned into the pEGFP-N3 vector.

2.8.4. Western blot assay

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton™ X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and protease inhibitor cocktail) and clarified through centrifugation at 14,000 $\times g$ for 20 min at 4 °C. Subsequently, the protein samples were subjected to Western blotting with the indicated antibodies.

2.8.5. Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde for 1 h at room temperature (RT), permeabilized using 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 min, and incubated in blocking reagent (5% normal FBS in PBS) for 1 h. The cells were incubated with primary antibody against MAP2, NF-L, acetylated α-tubulin, and α-tubulin (Mouse Anti-MAP2, Rabbit Anti-acetylated α-tubulin, Rabbit Anti-NF-L, and Mouse Anti-α-tubulin) at 4 °C overnight, as well as with secondary antibody (Alexa Fluor 488, Goat Anti-mouse; Alexa 568, Goad Anti-rabbit) at RT for 1 h. For nuclear counterstaining, the cells were incubated with DAPI solution (300 nM in PBS) for 5 min at RT, followed by observation under a fluorescence microscope (Eclipse Ti—S, Nikon, Tochigi, Japan).

3. Results

3.1. Clinical features of Korean patients with HSP

Our cohort comprised 132 and 34 affected and unaffected individuals, respectively, from 104 Korean families with 42 familial and 62 sporadic samples. As shown in Table 1, 82 probands in the families were genetically defined as HSP-related genes; their inheritance modes were 39 *de novo*, 33 AD, and 10 AR. 132 patients presented with features consistent with pure ($n = 111$) and complicated ($n = 21$) HSP. There were 50 (37.9%) women and 82 (62.1%) men. The age of onset (AAO) averaged 30.3 years, ranging from infancy to adulthood. The average SPRS score, representing the severity of HSP, was 16.7. Most patients reported lower extremity weakness and spasticity; however, none

Table 1
Clinical features of the Korean patients with HSP.

		Number
Family (affected/unaffected)		104 (132/34)
Family history (%)	Familial	42 (40.4)
	Sporadic	62 (59.6)
Genetically determined proband		82
Mode of inheritance (%)	*De novo*	39 (47.6)
	Autosomal dominant	33 (40.2)
	Autosomal recessive	10 (12.2)
Affected (patient)		132
Gender (%)	Female	50 (37.9)
	Male	82 (62.1)
Age of onset (mean (SD))	Total	30.3 (16.95)
	Early age of onset (< 35)	16.4 (10.5)
	Late age of onset (≥ 35)	45.1 (7.33)
Diagnosis (%)	Pure	111 (84.1)
	Complicated	21 (15.9)
SPRS (mean (SD))		16.7 (10.25)
LE weakness (%)	Yes	93 (70.5)
	No	38 (28.8)
	Unknown	1 (0.7)
LE spasticity (%)	Yes	127 (96.2)
	No	5 (3.8)
LE sensory impairment (%)	Yes	23 (17.4)
	No	108 (81.8)
	Unknown	1 (0.8)
Bladder symptom (%)	Yes	52 (39.4)
	No	80 (60.6)
Additional symptoms	Amyotrophy	2
	Ataxia	8
	Bulbar symptoms	10
	Carpal tunnel syndrome	2
	Cataract	1
	Cognitive impair	1
	Constipation	1
	Decreased vibration on leg	1
	Deep tendon reflex increased	1
	Dysarthria	1
	Mental retardation	2
	Narrow corpus callosum	1
	Optic atrophy	2
	Peripheral neuropathy	6
	Pes cavus	8
	Scoliosis	1
	Sensorimotor polyneuropathy	1

SPRS, Spastic Paraplegia Rating Scale; LE, Low extremity; SD, standard deviation.

reported sensory impairment. Further, 40% of the patients reported bladder-related symptoms. Table S1 summarizes the more detailed clinical information.

3.2. Genetic variation profiling of Korean patients with HSP

As shown in Fig. 1, to identify the genetic defects in Korean patients with HSP, we generated and analyzed WES and direct-sequencing obtained from 104 HSP families. We identified 88 genetic variants in 54 genes from 82 (78.8%) probands after excluding 22 probands with genetically undefined HSP; subsequently, they were assigned as the 54 kHSP gene set. Based on previously reported HSP-related genes, the gene set was classified into three mutation groups: i) 53 variants within 19 known HSP genes (46 probands); ii) 26 variants within 26 putative candidate HSP genes (27 probands), involving in HSPome [20]; and iii) 9 variants within 9 neuronal-related genes (9 probands).

Among the 19 known HSP genes, *SPAST* (41%) exhibited the highest frequency, followed by *ALDH18A1*, *ATL1*, *ATP2B4*, *KIF5A*, *REEP1*, *SPG7*, *SPG11*, and *WASHC5* (Fig. 2A). Our mutations identified in the 14 known HSP genes, including *ALDH18A1*, *ALS2*, *AP5Z1*, *ATP2B4*, *BSCL2*, *CAPN1*, *CYP7B1*, *DDHD1*, *KIDINS220*, *PLP1*, *SPART*, *SPG7*, *SYNE1*, and *WASHC5*, were firstly reported in Korean patients with HSP; moreover, eight variants in the *DDHD1*, *PLP1*, *SPAST*, and *SPG7* genes were novel. The 53 variants in the known HSP genes included previously reported 35 AD-HSP, 10 AR-HSP, and one X-linked HSP variant (Fig. 2B). In the 54 kHSP gene set, there was missense (66%), Ins/Del/Dup (16%), splicing (12%), and nonsense (6%) mutation types (Fig. 2C), which is consistent with those of known HSP genes.

We evaluated the pathogenicity of our variants based on the ACMG

classification. We identified 15 pathogenic variants located within five known HSP genes, including *CAPN1*, *REEP1*, *SPAST*, *SPG7*, and *SPG11*, as well as within one putative candidate HSP gene (*PHF3*) and one neuronal-related gene (*NFIA*) (Table 2). Among these variants, four were compound heterozygous, five were in the splicing site, five were frameshift deletions, and three were nonsense mutations. Additionally, 46% of these variants were located in *SPAST*. The 31 likely pathogenic variants are detected within 9 known HSP genes, including *ALDH18A1*, *ATL1*, *CYP7B1*, and *SPAST*, as well as seven putative candidate HSP genes, including *AMPD3*, *CNGB3*, and *PDE4B* (Table 3). We observed nine short deletions and one duplicated mutation. Furthermore, using *in silico* prediction tools, we assigned 24 damaging variants from 42 uncertain significant variants (Table S2). These results suggest that our kHSP gene set may contain HSP-causative variants. This information of genetic variants in the kHSP gene set is presented in detail in Table S3.

AAO analysis based on our and previously reported Korean patients with HSP reported two high peaks at 0–10 and 31–40 years (Fig. S1A), which coincides with data on global patients with HSP showing bimodal distribution [7,13]. The AAO in patients with *SPAST* is centralized at 35 years, which is consistent with late adulthood onset reported in a previous study (Fig. S1B) [22]. Patients with *ATL1* and *REEP1* have an early AAO. The AAO for patients with *ALDH18A1* is 10–30 years. Taken together, these results suggest that the genetic spectrum and variation of HSP-related genes in Korean patients with HSP are diverse.

3.3. Molecular function and interaction network of the kHSP gene set

We scrutinized the functional annotation and interaction networks of the genes to elucidate the relationships among the kHSP genes. GO
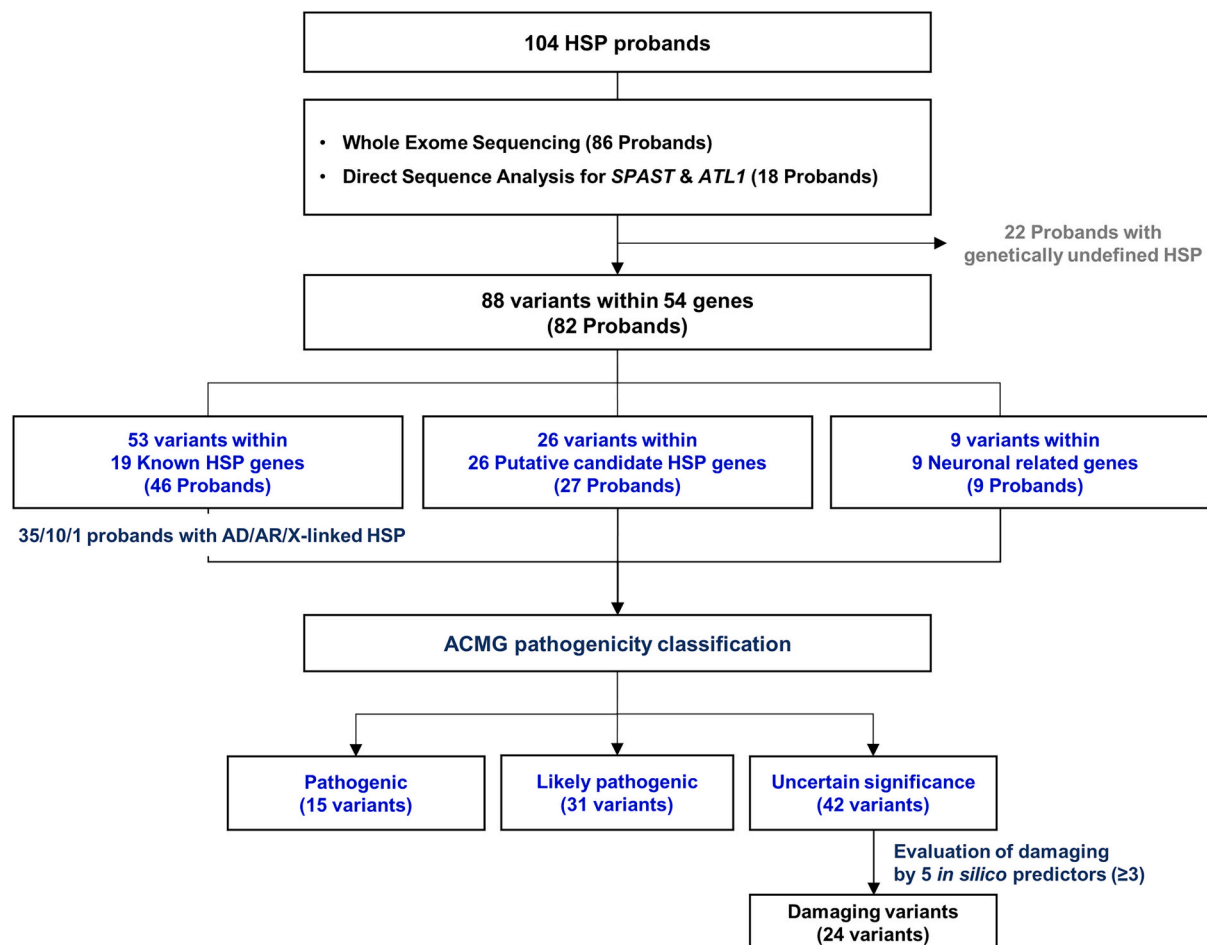

Fig. 1. Workflow for identifying HSP-related causative genetic variants.
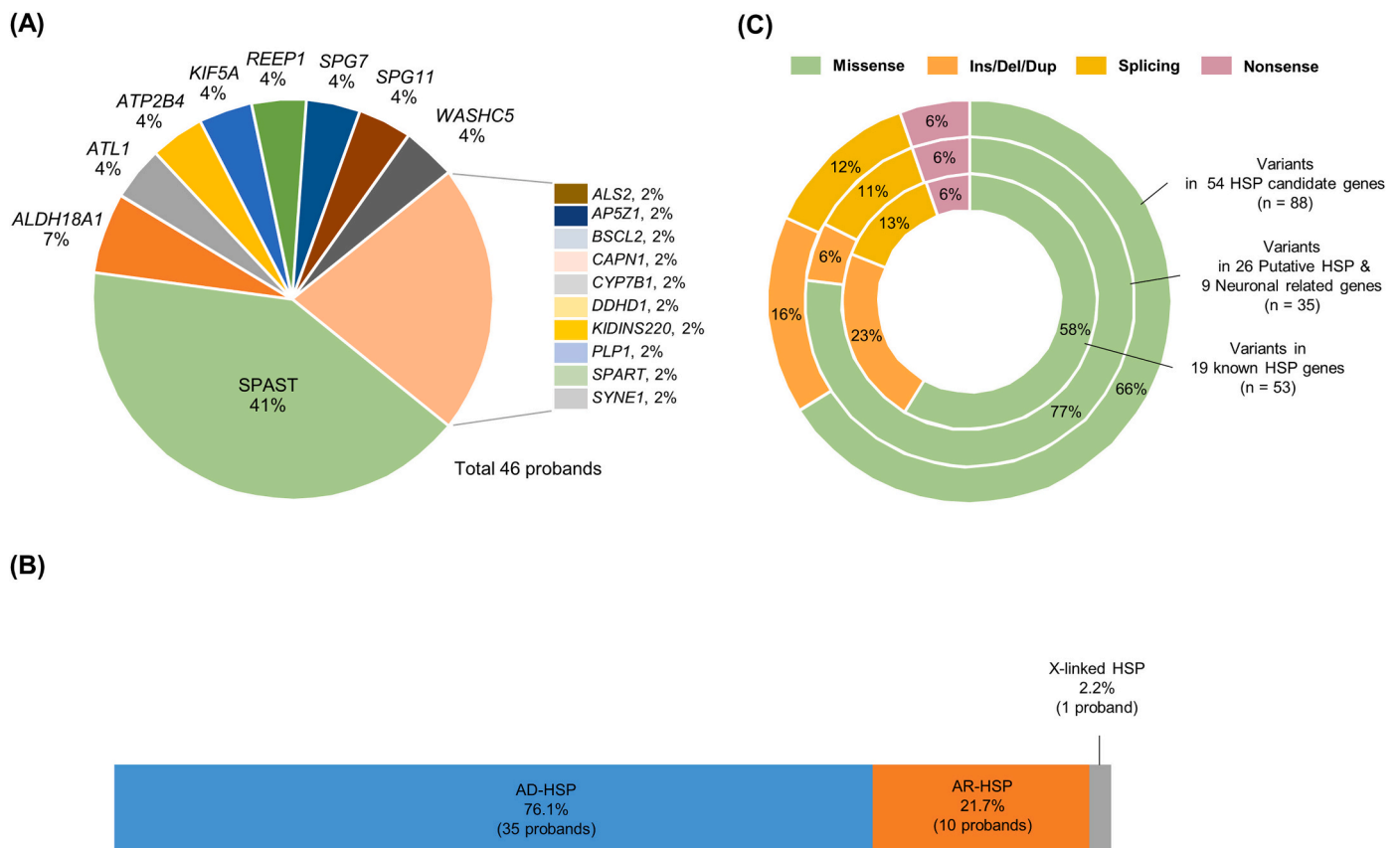
(A)

(B)

(C)

Fig. 2. Statistics of genetic variations in the 54 kHSP gene set (A-B) Frequency (A) and previously reported inheritance patterns (B) of the known HSP genes in 46 HSP probands. (C) Genetic variant types in the 54 kHSP gene set. The number in parentheses represents the number of probands.

analysis revealed that the genes were significantly involved in 23 cellular components, including polymeric cytoskeletal fiber and the endoplasmic reticulum tubular network (Fig. 3A and Table S4A). Furthermore, the genes were enriched in 168 biological processes, including endoplasmic reticulum organization and the cellular response to epinephrine stimulus. Regarding molecular function, they were enriched in 46 functions involving bindings (microtubule and actin), microtubule motor activities, and enzymes activities (steroid hydroxylase and lipase). Furthermore, molecular function analysis through ingenuity pathway analysis revealed that the gene set accounted for development, organismal injury/abnormalities, behavior, neurological disease, and lipid/amino acid/nucleic acid metabolism (Fig. 3B). Additionally, the genes were distributed in the phenotype ontology, including lower limb muscle weakness, spastic gait, vibratory sensation, spastic paraplegia, and motor neurons (Fig. 3C and Table S4B).

Furthermore, network analysis revealed genes related to a network of nervous system development and function, including the manifestations of neurological diseases and hereditary disorders (Fig. 3D).

Table 2

Summary of HSP probands with pathogenic variations based on the ACMG classification.

ID	# of affected	Gene symbol	Nucleotide change	Amino acid change	ACMG classification	Allele zygosity	Inheritance	Group
F42-P01	1	*CAPN1*	NM_001198868.2:c.1015C > T/ NM_001198868.2:c.1142C > T	p.Arg339Ter/p.Ala381Val	P/VUS	c_Ht	AR	Known HSP
F39-P01	2	*REEP1*	NM_022912.3:c.303+2T > C	Splicing site	P	Ht	AD	Known HSP
F04-P01	3	*SPAST*	NM_014946.4:c.1031T > A	p.Ile344Lys	P	Ht	AD	Known HSP
F07-P01	4	*SPAST*	NM_014946.4:c.1098 + 1G > A	Splicing site	P	Ht	AD	Known HSP
F08-P01	1	*SPAST*	NM_014946.4:c.570delC	p.Arg191AlafsTer*5	P	Ht	AD	Known HSP
F11-P01	1	*SPAST*	NM_014946.4:c.1245 + 1G > A	Splicing site	P	Ht	AD	Known HSP
F12-P01	4	*SPAST*	NM_014946.4:c.911dupC	p.Thr305TyrfsTer*6	P	Ht	AD	Known HSP
F14-P01	6	*SPAST*	Exon2–6deletion		P	Ht	AD	Known HSP
F25-P01	1	*SPG7*	NM_003119.4:c.1192C > T/ NM_003119.4:c.2153T > C	p.Arg398Ter/p.Leu718Pro	P/LP	c_Ht	AR	Known HSP
F33-P01	1	*SPG11*	NM_025137.4:c.733_734delAT/ NM_025137.4:c.5866 + 1G > A	p.Met245ValfsTer*2/ Splicing site	P/P	c_Ht	AR	Known HSP
F34-P01	1	*SPG11*	NM_025137.4:c.5410_5411delTG/ NM_025137.4:c.3291 + 1G > T	p.Cys1804ProfsTer*25/ Splicing site	P/P	c_Ht	AR	Known HSP
F54-P01	1	*PHF3*	NM_001370349.2:c.5536 > T	p.Arg1846Ter	P	Ht	*De novo*	Putative HSP
F77-P01	1	*NFIA*	NM_001134673.4:c.1051C > T	p.Arg351Ter	P	Ht	*De novo*	Neuronal

ACMG, American College of Medical Genetics; Ht, heterozygous; c_Ht,compound heterozygous; P, pathogenic; VUS, variants of uncertain significance; AD, autosomal dominant; AR, autosomal recessive.

Table 3
Summary of HSP probands with likely pathogenic variations based on the ACMG classification.

ID	# of affected	Gene symbol	Nucleotide change	Amino acid change	ACMG classification	Allele zygosity	Inheritance	Group
F28-P01	3	*ALDH18A1*	NM_002860.4:c.755G>A	p.Arg252Gln	LP	Ht	AD	Known HSP
F29-P01	1	*ALDH18A1*	NM_002860.4:c.1315G>A	p.Gly439Ser	LP	Ht	*De novo*	Known HSP
F30-P01	1	*ALDH18A1*	NM_002860.4:c.34C>G	p.Pro12Ala	LP	Ht	*De novo*	Known HSP
F02-P01	2	*ATL1*	NM_015915.5:c.715C>T	p.Arg239Cys	LP	Ht	AD	Known HSP
F03-P01	2	*ATL1*	NM_015915.5:c.536C>A	p.Ser179Tyr	LP	Ht	AD	Known HSP
F35-P01	2	*BSCL2*	NM_001122955.4:c.461C>T	p.Ser154Leu	LP	Ht	AD	Known HSP
F23-P01	1	*CYP7B1*	NM_004820.5:c.1229C>T/ NM_004820.5:c-.1403delC	p.Pro410Leu/p. Thr468IlefsTer*5	VUS/LP	c_Ht	AR	Known HSP
F38-P01	1	*DDHD1*	NM_030637.3:c.2525A>G/ NM_030637.3:c.1571_1572delTT	p.Tyr842Cys/p. Phe524Ter	VUS/LP	c_Ht	AR	Known HSP
F31-P01	1	*KIF5A*	NM_004984.4:c.967C>T	p.Arg323Trp	LP	Ht	*De novo*	Known HSP
F32-P01	1	*KIF5A*	NM_004984.4:c.839G>A	p.Arg280His	LP	Ht	AD	Known HSP
F01-P01	1	*PLP1*	NM_000533.5:c.833G>C	p.Ter278Serext*14	LP	Hemi	*De novo*	Known HSP
F05-P01	2	*SPAST*	NM_014946.4:c.1196C>G	p.Ser399Trp	LP	Ht	AD	Known HSP
F06-P01	2	*SPAST*	NM_014946.4:c.1135_1140del	p.Leu379_Leu380del	LP	Ht	AD	Known HSP
F09-P01	1	*SPAST*	NM_014946.4:c.1457C>T	p.Thr486Ile	LP	Ht	*De novo*	Known HSP
F10-P01	1	*SPAST*	NM_014946.4:c.1210_1212delTTT	p.Phe404del	LP	Ht	AD	Known HSP
F13-P01	1	*SPAST*	NM_014946.4:c.1414-2A>G	Splicing site	LP	Ht	*De novo*	Known HSP
F15-P01	1	*SPAST*	NM_014946.4:c.1712_1728 + 84del	–	LP	Ht	AD	Known HSP
F17-P01	1	*SPAST*	NM_014946.4:c.1114A>G	p.Arg372Gly	LP	Ht	AD	Known HSP
F18-P01	1	*SPAST*	NM_014946.4:c.1011del	p.Ala338Leufs*7	LP	Ht	AD	Known HSP
F19-P01	1	*SPAST*	NM_014946.4:c.1114A>G	p.Arg372Gly	LP	Ht	AD	Known HSP
F20-P01	1	*SPAST*	NM_014946.4:c.67_85dup	p.Leu29Glnfs*25	LP	Ht	AD	Known HSP
F21-P01	1	*SPAST*	NM_014946.4:c.1082C>T	p.Pro361Leu	LP	Ht	AD	Known HSP
F22-P01	1	*SPAST*	NM_014946.4:c.1112T>G	p.Leu371Arg	LP	Ht	AD	Known HSP
F27-P01	1	*WASHC5*	NM_014846.4:c.2086G>A	p.Gly696Ser	LP	Ht	*De novo*	Known HSP
F72-P01	2	*AMPD3*	NM_001172431.1:c.1240C>T	p.Arg414Cys	LP	Ht	AD	Putative HSP
F69-P01	1	*CNGB3*	NM_019098.5:c.1898A>G	p.Asp633Gly	LP	Ht	*De novo*	Putative HSP
F58-P01	1	*CYP2W1*	NM_017781.3:c.173A>C	p.Glu58Ala	LP	Ht	AD	Putative HSP
F67-P01	1	*GALNS*	NM_001323544.2:c.137_138del	p.Glu46AspfsTer*5	LP	Ht	*De novo*	Putative HSP
F59-P01	1	*KRT6A*	NM_005554.4:c.1021T>C	p.Tyr341His	LP	Ht	*De novo*	Putative HSP
F71-P01	1	*OBSL1*	NM_015311.3:c.2135-3_2135-2delCA	Splicing site	LP	Ht	AD	Putative HSP
F65-P01	1	*PDE4B*	NM_001297442.2:c.48 + 1G>T	Splicing site	LP	Ht	*De novo*	Putative HSP

ACMG, American College of Medical Genetics; Ht, heterozygous; c_Ht, compound heterozygous; Hemi, hemizygous; P, pathogenic; LP, likely pathogenic; VUS, variants of uncertain significance; AD, autosomal dominant; AR, autosomal recessive.

Specifically, nine neuronal-related genes were directly or indirectly linked to known- and putative candidate-HSP genes as follows: *NFIA* links to *GJC3*, *ATP2B4*, *PLP1*, *SETX*, and *SPART via UBC*; *KIF19* links up to *SPG7* and *SPART via ZNF7* and *MAPK6*, as well as to *AP5Z1 via TBR1XL1*; *CELSR2*, *COBL*, and *JAG1* link to *WASHC5*, *ALDH18A1* and *PHF3 via VIRMA*; *RUNX3* links to *REEP1*; *ADGRB1* links to *CNGB3 via NEK4*; and *CCDC88A* and *EN1* link to *ATL1*, *CAPN1*, *PLP1*, and *SPART via HTT*. Table S5 lists the 118 genes in the interaction network in detail. These results suggest extensive connections between our kHSP gene set, which is suggestive of an important role in HSP progression.

3.4. Genetic spectrum of known HSP genes between ethnic groups

Comparison among known HSP genes in different ethnic groups revealed that the frequency of *SPAST*, *ATL1*, *SPG11*, *SPG7*, and *CYP7B1*, which are the five most common HSP genes, is very high (> 80%) in CN, FC, and HU groups, but very low (32%) in the IT group (Fig. 4A). As expected, *SPAST* variants exhibited the highest frequency in all groups except IT. *SPG11* in CN and *SPG7* in HU exhibited higher frequencies compared with those of other groups. There were no *SPG7* and *ATL1* variants in the CN and IT groups, respectively.

Furthermore, genetic variation analysis revealed that missense and Ins/Del/Dup types had a higher frequency than the splicing and nonsense types (Fig. 4B). In *SPAST*, there were various missense variants in FC and Ins/Del/Dup in CN. However, there is a similar frequency of these variant types in Koreans. In *ATL1*, only missense and Ins/Del/Dup types were observed in all ethnic groups other than IT. Notably, the Ins/Del/Dup type in *SPG11* exhibited a high frequency.

Frequency analysis of commonly known HSP genes revealed that

Korean and IT patients had wider spectrums compared with other ethnic groups (Fig. 4C). Specifically, *ALDH18A1* (7%) and *KIF1A* (8.5%) had higher frequencies in Korean and IT patients, respectively. Unlike in the other ethnic groups, *ATP2B4*, *KIF5A*, and *BSCL2* were mainly observed in Korean patients. *ADAR*, *DDHD2*, and *KIF1A* were mainly observed in IT while *FARS2* and *GBA2* were mainly observed in CN. Taken together, these results indicate the diversity of the genetic spectrum and variation of known HSP genes across ethnic groups, which is indicative of differences in the mode of HSP progression according to ethnicity.

3.5. Known HSP genes not previously identified in Korean patients with HSP

Among the firstly identified four known HSP genes in Korean patients with HSP, variant analysis revealed that c.833G > C on *PLP1* was inherited as a *de novo* mutation in the X-linked recessive (hemizygous) mode; c.1192C > T and c.2153 T > C on *SPG7* as an AR inheritance (compound heterozygous); c.755G > A on *ALDH18A1* and c.461C > T on *BSCL2* as an AD inheritance (Fig. 5A–B). The altered amino acids showed high evolutionary conservation in multiple species, which indicated important roles in biological progress (Fig. 5C).

Protein modeling analysis revealed that p.*278Sext*14 mutation on *PLP1* would lead to peptide extension, which would destabilize and damage the protein function within the membrane, indicating that it affects myelin sheath formation. The p.R398* mutation of *SPG7* would terminate protein synthesis, which would yield an incomplete polypeptide. The p.L718P mutation, which is a substitution to proline, would distort the secondary structure in the middle of the alpha-helical region involved in the hexameric arrangement of metalloprotease and lead to
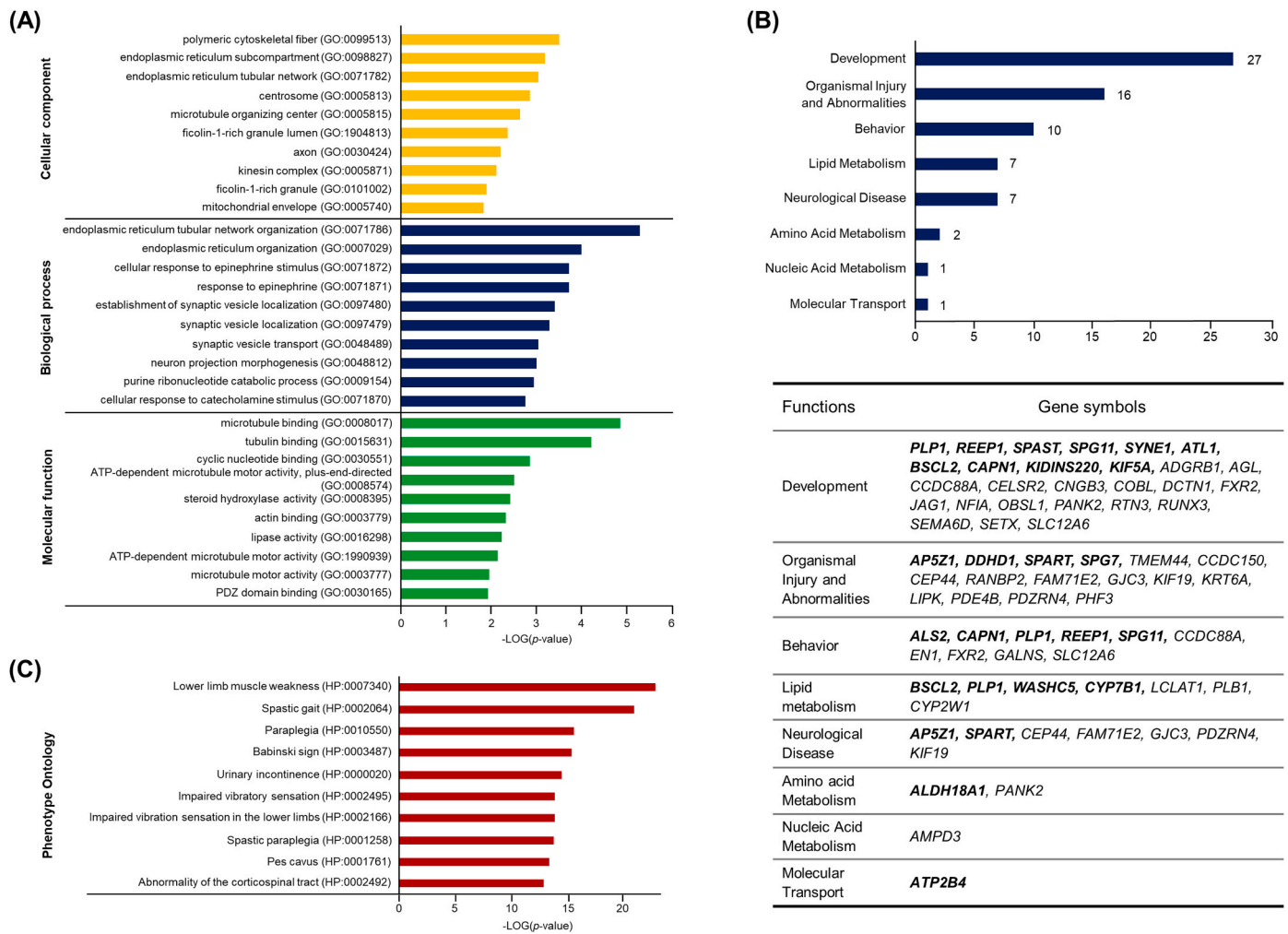
(A)

(B)

Functions	Gene symbols
Development	***PLP1, REEP1, SPAST, SPG11, SYNE1, ATL1, BSCL2, CAPN1, KIDINS220, KIF5A,*** ADGRB1, AGL, CCDC88A, CELSR2, CNGB3, COBL, DCTN1, FXR2, JAG1, NFIA, OBSL1, PANK2, RTN3, RUNX3, SEMA6D, SETX, SLC12A6
Organismal Injury and Abnormalities	***AP5Z1, DDHD1, SPART, SPG7,*** TMEM44, CCDC150, CEP44, RANBP2, FAM71E2, GJC3, KIF19, KRT6A, LIPK, PDE4B, PDZRN4, PHF3
Behavior	***ALS2, CAPN1, PLP1, REEP1, SPG11,*** CCDC88A, EN1, FXR2, GALNS, SLC12A6
Lipid metabolism	***BSCL2, PLP1, WASHC5, CYP7B1,*** LCLAT1, PLB1, CYP2W1
Neurological Disease	***AP5Z1, SPART,*** CEP44, FAM71E2, GJC3, PDZRN4, KIF19
Amino acid Metabolism	***ALDH18A1,*** PANK2
Nucleic Acid Metabolism	AMPD3
Molecular Transport	***ATP2B4***

(C)

Fig. 3. Biological ontology and network analysis of the 54 kHSP gene set. (A) Gene Ontology (GO) analysis. Top 10 significantly enriched GO [-LOG(*p*-value)] terms in the 54 kHSP gene set for cellular component, biological process, and molecular functions. (B) The biological function of the kHSP gene set was determined using ingenuity pathway analysis (IPA). Numbers (upper) and lists (lower) of genes in each category are presented. Bold indicates known HSP genes. (C) Phenotype Ontology (PO) analysis. Top 10 significantly enriched PO [-LOG(*p*-value)] terms in the 54 kHSP gene set. (D) Interaction network of the kHSP gene set. A dotted line indicates interactions between neuronal-related genes and known/putative candidate HSP genes. Genes shown in red, blue, and yellow indicate known HSP, putative candidate HSP, and neuronal-related genes, respectively.

the disruption of the functional architecture (Fig. 5D, left). Since the p. S154L mutation in *BSCL2* is involved in protein glycosylation, it would hinder protein glycosylation and cause protein malfunction, which results in seipinopathy (Fig. 5D, right) [26]. These results are indicative of the larger genetic variability in the Korean HSP population compared with that indicated by previous reports; moreover, they expand the genetic variability in HSP disease progression.

3.6. SPAST and REEP1 mutants with unknown function induce neurite abnormality

Among the variants with unknown function in our known HSP genes, we selected *SPAST* and *REEP1* gene mutants and conducted functional studies of underlying neuronal differentiation processes to elucidate their pathogenic function. The identified *SPAST* mutations were mostly located in the AAA domain (Fig. 6A). The clinical significance and pathogenicity of the *SPAST* mutants, including p.S399W, p.T486I, p. F404del, and p.L379_L380del, are mostly unknown.

To explore the functional effects of the mutants, we transfected HEK293 cells with four mutant *SPAST* plasmids. In contrast to the *SPAST*-WT, which exhibited punctate formation, in-frame deletion mutations (p.F404del and p.L379_L380del) exhibited cytosolic

localization while missense mutations (p.S399W and p.T486I) exhibited a filamentous pattern in cells (Fig. 6B). Since many *SPAST* pathogenic mutants are of the filamentous type and bind with microtubules [25,27,28], the mutant-caused localization changes could be pathogenic. Compared with the wild type, *SPAST* mutation caused abnormal neurite outgrowth and inhibited the expression of neuron-specific proteins in differentiated SH-SY5Y cells (Fig. 6C). Notably, *SPAST*-S399W and T486I-treated cells exhibited decreased microtubule acetylation; contrastingly, *SPAST*-F404del and L379_L380del cells exhibited increased α-tubulin acetylation (an indicator of stable microtubule) (Fig. 6D). Similar findings were yielded by Western blot analysis (Fig. 6E). Furthermore, studies with *REEP1*-D116N revealed that it showed dot formation, which was unlikely to be observed with *REEP1*-WT (Fig. S2A) and caused neurite abnormalities (Fig. S2B–D). These results suggest that *SPAST* and *REEP1* mutants may have pathogenic effects involving impaired neuronal outgrowth, which eventually causes HSP.

4. Discussion

HSPs are a large group of inherited neurologic disorders that comprise the most clinically and genetically heterogeneous human
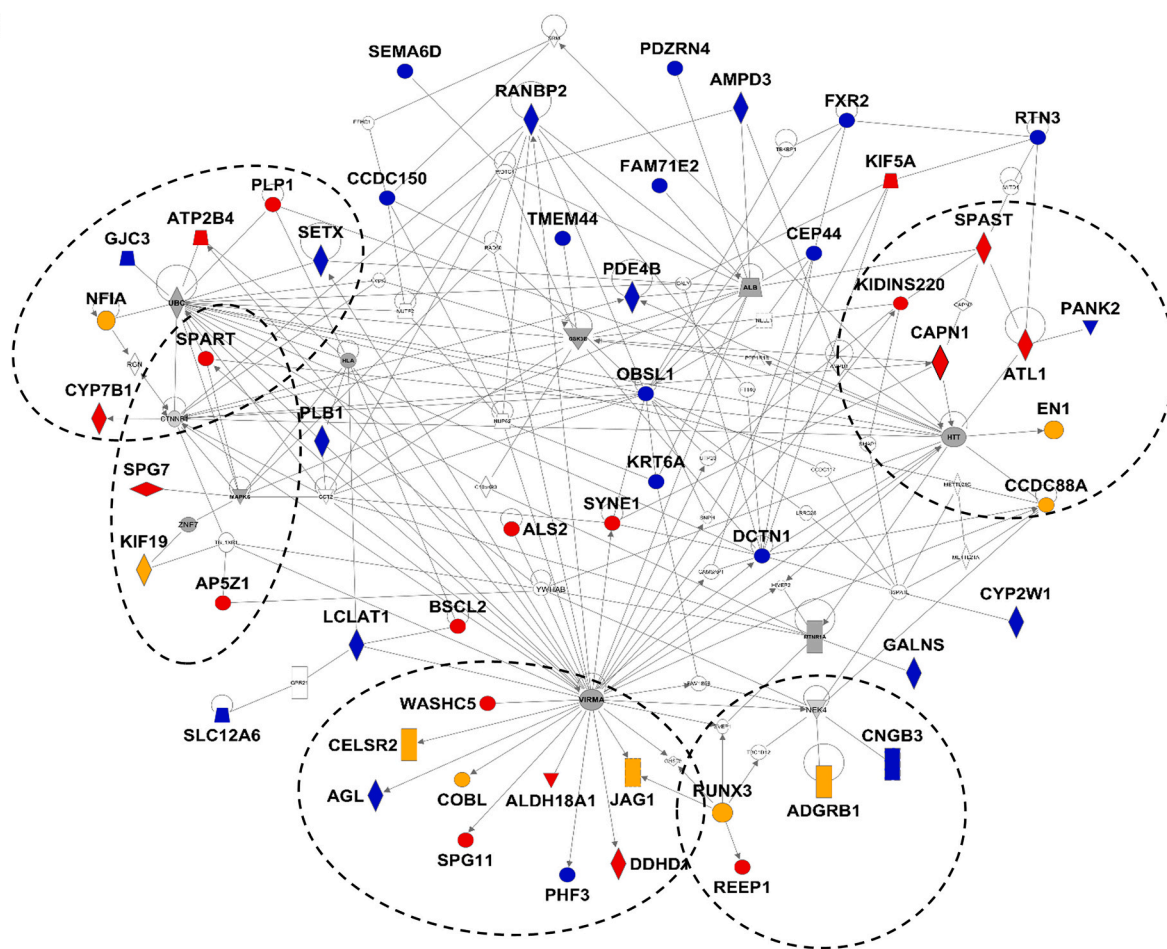
(D)

Fig. 3. (*continued*).

diseases. Several studies have reported novel causative genes of HSPs; however, there is considerable clinical overlap between patients with different mutations, which impedes genetic diagnosis [2]. In this study, we identified 88 genetic variants and the 54 kHSP gene set from 104 Korean HSP probands with well-defined clinical information to facilitate accurate genetic diagnosis and a mechanistic understanding of HSP. The HSP genes were closely linked to a network related to HSP progression; moreover, the genetic spectrum and variations differed across ethnic groups. These findings can facilitate the discovery of potential diagnostic and therapeutic targets for HSP.

Using WES data analysis, we identified a 54 kHSP gene set, which contained 19 known HSP genes, 26 putative candidate HSP genes, and 9 neuronal-related genes. To efficiently identify putative candidate HSP genes, we compared our genes with those in HSPome, which contains 589 known and possible HSP genes through network propagation [20]. All of the putative HSP genes have a single heterozygous variant with AD or *de novo* inheritance (Table S3B and Fig. S3), while the putative genes from the HSPome have homozygous/compound heterozygous variants, with most of them having consanguineous/recessive inheritance. Contrary to the report of the HSPome, single heterozygous variants (p. Val36Glu and p.Met40Arg) in *REEP2* were also reported as AD and *de novo* in the two HSP families, respectively [29,30]. In addition, the p. Lys508Thr variant in *BICD2* has been reported as AD in one HSP family, though it is a very rare case [31]. These findings suggest that our putative candidate HSP genes with a single heterozygous variant could be potential HSP-related genes, which may eventually aid in the genetic diagnosis of HSP. Moreover, the 19 known HSP genes showed highly heterogeneous ins/del/dup, splicing, and nonsense mutations compared with putative candidate HSP and neuronal genes, which indicates a

higher damaging effect of known HSP genes. In particular, we identified and reported 14 known HSP genes, with a very high frequency of 74%, in Korean patients with HSP for the first time; four of the 25 variants were novel.

Genotype-phenotype features revealed that our HSP cohort comprised mainly patients with the AD form regardless of the HSP type; contrastingly, a previous study reported that pure and complicated HSPs are usually inherited in an AD and AR manner, respectively [32]. Furthermore, there was a higher prevalence of *SPAST* (53%) among females, which is inconsistent with a previous report [33]. Notably, *ALDH18A1* patients presented with pure HSP, and with the second-highest frequency in our cohort; contrastingly, it has been previously reported to cause complicated HSP as an extremely rare HSP gene [4]. Consistent with previous reports, *SPG7*-mutant patient with p.V549M presented with pure HSP of the AD type [34], a extremely rare case; contrastingly, *ATP2B4* and *DDHD1* patients presented with the AD type [4]. Additionally, *BSCL2* patients presented with complicated HSP characterized by neuropathy and pes cavus [4]; moreover, *ATL1* patients presented with pure-HSP with early-onset, similar to previous reports [4]. *KIF5A* patients with p.R323W and p.R280H were found to present with pure and complicated HSPs, respectively, unlike previous reports [35]. As shown in Table S6, *WASHC5* patients presented with not only pure HSP but also with complicated HSP, unlike in previous reports in which the patients mainly presented with pure HSP [4]. Furthermore, *CAPN1* patients with compound heterozygous (p.R339X/p.A381V) presented with pure HSP; a previous study reported that a patient with compound heterozygous (p.A381V/p.Q155X) presented with complicated HSP while a patient with homozygous (p.R339X) presented with pure and complicated HSP [36–38]. Cumulatively, these results confirm
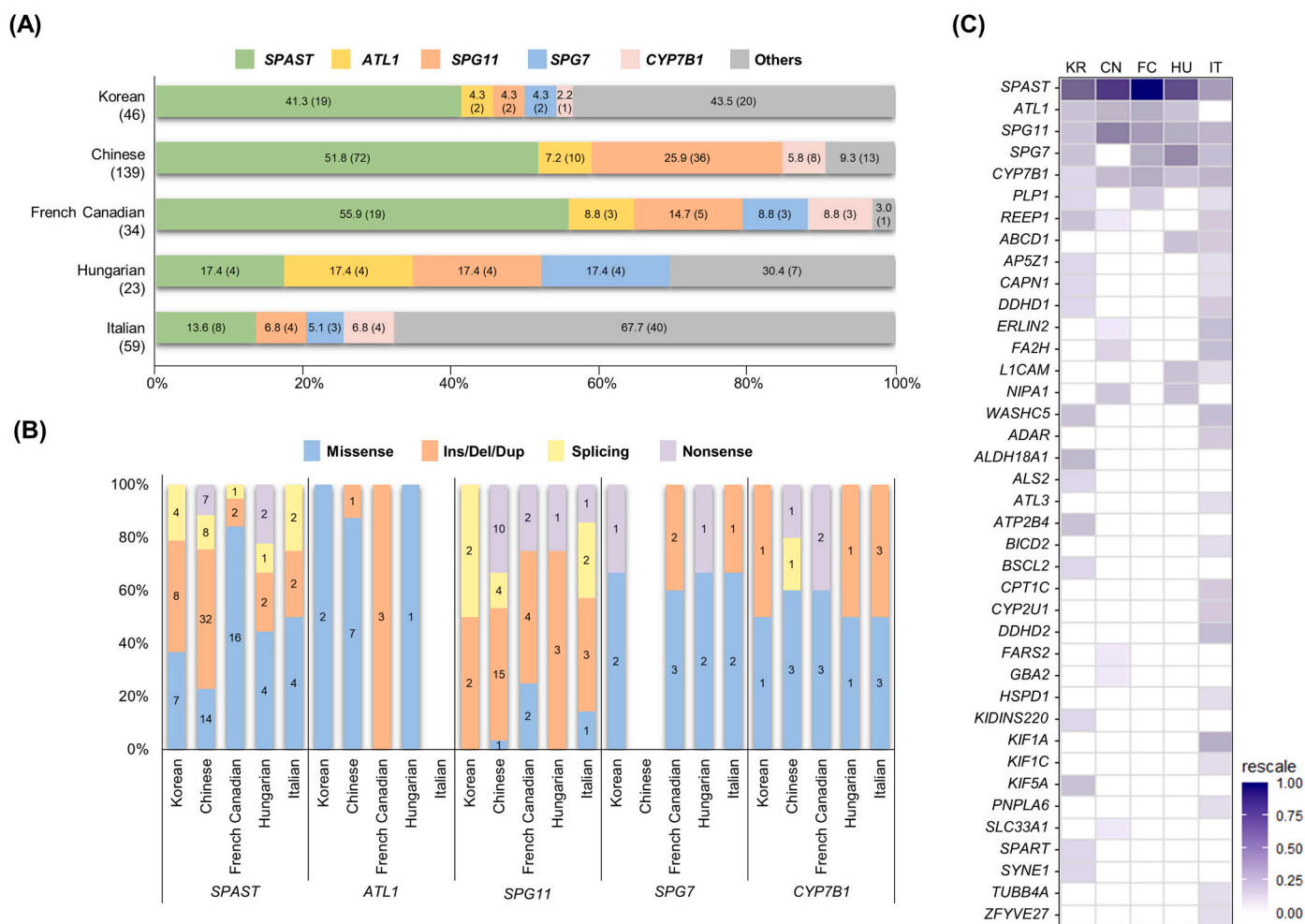
(A)

(B)

(C)

Fig. 4. Ethnic comparison of known HSP genes. (A) Frequency of the top-ranked known HSP genes (*SPAST*, *ATL1*, *SPG11*, *SPG7*, and *CYP7B1*) in KR, CN, FC, HU, and IT. The number in parentheses means the number of probands. (B) The ratio of variant types of top-ranked known HSP genes in each ethnic group. The numbers indicate the number of variant types. (C) Heat map for frequency of reported total known HSP genes in each ethnic HSP cohort. KR, Korean; CN, Chinese; FC, French Canadian; HU, Hungarian; IT, Italian. KR data were derived from an analysis of our cohort results.

the clinical and genetic heterogeneity of HSP, and therefore may facilitate the confirmation of gene mutations in individual families, as well as improve the genotype-phenotype correlation.

The identified genetic variations significantly affected the HSP pathogenicity. Based mainly on the ACMG classification and partially on the *in silico* prediction tools, approximately 78% of kHSP genes have 15 pathogenic variants, 31 likely pathogenic variants, and 24 damaging variants. Specifically, *SPAST* has four novel and 14 known variants, including six pathogenic, 11 likely pathogenic, and one uncertain significant type, which were not assigned PS3 items related to functional data in the ACMG classification. Similarly, the clinical significances of these variants were also assigned 'VUS' or 'Not reported' in the ClinVar database, due to the absence of functional analysis. To elucidate pathogenic function of the variants in our known HSP genes, we selected mutants of *SPAST* and *REEP1* genes, the main causes of HSP, and conducted functional studies related to the neuronal differentiation processes. Among *SPAST* variants, four variants, including *SPAST*-S399W, T486I, F404del, and L379_L380del, are functionally unknown while the other mutants can be predicted to code too-short proteins or to lead to total deletion of the AAA domain, indicating protein malfunction. *In vitro* studies using four variants revealed remarkable pathogenic effects on neuronal differentiation, including neurite abnormalities and impaired neuronal differentiation, which is supported by previous

studies [39,40]. Notably, two in-frame deletion mutants may impair neuronal differentiation through hyper-stabilization of microtubules [25,41], with two other missense mutants altering microtubule stability in a contrasting manner, *i.e.*, decreasing α-tubulin acetylation, which is consistent with previous findings obtained using the *SPAST* mouse model [42]. Furthermore, *REEP1*-D116N also exhibited pathogenic effects showing exhibiting localization changes and abnormal neurite outgrowth, similar to those of other previously reported *REEP1* mutations [43]. Furthermore, protein modeling of three proteins predicted the pathogenic effects of their variants. Specifically, c.833G > C on *PLP1* affects myelin sheath formation through peptide extension while c.461C > T on *BSCL2* causes seipinopathy by hindering glycosylation [26]. Furthermore, *SPG7* leads to protein malfunction through an incomplete polypeptide *via* c.1192C > T and disruption of the protein secondary structure *via* c.2153 T > C. These results suggest that our identified variants may have pathogenicity that promotes HSP progression.

In the network analysis, 54 kHSP genes were found to be directly and indirectly linked on a network related to neurological diseases, skeletal and muscular disorders, and hereditary disorders. Specifically, *CELSR2*, *COBL*, *JAG1*, *RUNX3*, and *ADGRB1* (neuronal-related genes), as well as *AGL*, *PHF3*, *CNGB3*, *DCTN1*, *CYP2W1*, and *KRT6A* (putative candidate HSP genes), were connected with *WASHC5*, *SPG11*, *ALDH18A1*, *DDHD1*, *REEP1*, *SYNE1*, and *ALS2* (known HSP genes). Notably, they are
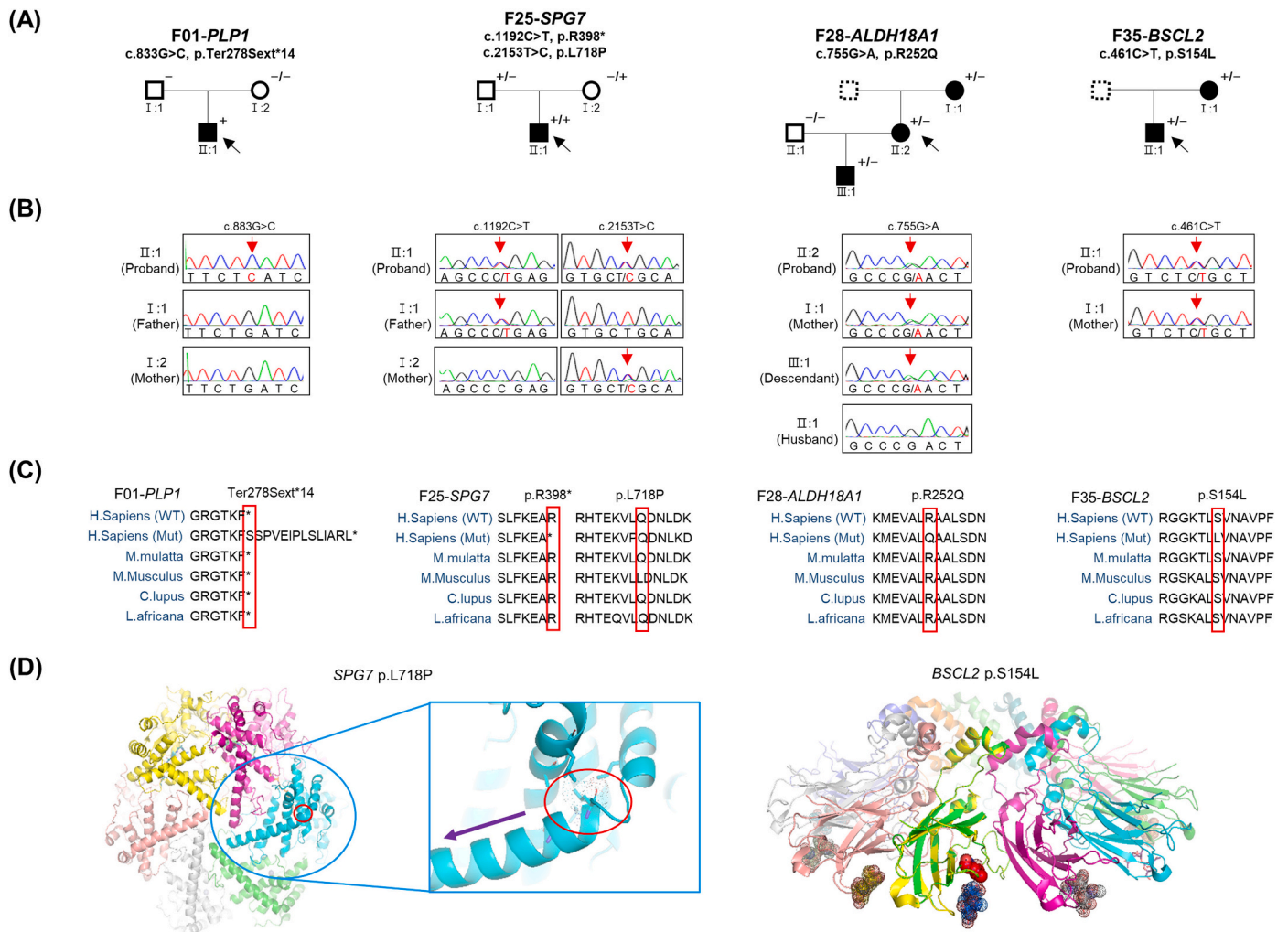
Fig. 5. Variants of the firstly identified known HSP genes in Korean patients with HSP. The known HSP genes (*PLP1*, *SPG7*, *ALDH18A1*, and *BSCL2*) that were first identified in our Korean HSP cohort are presented. (A) Family pedigrees. Filled and unfilled symbols present affected and unaffected individuals, respectively. The proband is indicated by the black arrow. Dotted lines indicate no samples. -, wild-type allele; +, mutant allele; −/−, wild-type; +/− or −/+, heterozygous; +/+, compound heterozygous; − or +, hemizygous. (B) Sanger sequencing data. The arrows indicate nucleotide mutation sites. (C) Conservation analysis of amino acid sequences. Variants are evolutionarily conserved in multiple species. (D) The three-dimensional (3D) structures of proteins. The red circle with sticks inside represents the residue 718 of SPG7 protein while the purple arrow indicates the destabilized helix due to the substitution (left). The residue 154 of BSCL2 protein is shown in red spheres, with glycosylation being shown in dots (right).

centralized on *VIRMA*, which is related to neuropsychiatric and neuro-degenerative disorders given the effects for synaptic and axonal function, indicating that *VIRMA*-connected genes may be crucially involved in the outgrowth and maintenance of axons in motor neurons [44], resulting in HSP progression. Additionally, enrichment analysis based on GO, phenotype ontology, and pathways revealed that kHSP genes share the same term on cellular components or molecular functions. For example, *CELSR*, *ATL1*, *ADGRB1*, and *PLP1* are related to axon development and cell morphogenesis involved in neuron differentiation. *JAG1* and *SYNE1* are associated with muscle cell differentiation. Our results indicate that neuronal-related genes, which are expressed in the cerebral cortex, spinal cord, and skeletal muscle [45], have strong potential as novel HSP genes. Moreover, they support the idea that rare genetic mutations may converge on a few key biological processes. Furthermore, our data suggest potential targets of HSP treatment. For example, *CYP2W1*, *LCLAT1*, and *PLB1*, which are linked to *BSCL2*, *CYP7B1*, *PLP1*, and *WASHC5*, as well as are related to the lipid metabolism, could be targeted by bypassing specific metabolic blocks. These results suggest that our kHSP gene set may facilitate the identification of novel candidate genes and HSP-related pathways.

The distribution frequency and mutation type of known HSP genes markedly differed across ethnic groups based on the largest reported datasets that represented each ethnic group. Specifically, Korean and IT groups showed a wide spectrum of known HSP genes; however, the CN, FC, and HU groups mostly presented with five known HSP genes. Korean patients had the highest frequency ($> 40\%$) of *SPAST* variants, which was similar to the FC, CN, and HU patients; however, other known genes are widely spread out, similar to IT patients. Similar to reports by previous Korean studies [46], Korean patients showed a lower frequency of *ATL1* variants. Notably, compared with the other ethnic groups, Korean patients had a high frequency of axonal transport genes, including *ALS2*, *KIDINS220*, and *KIF5A*, as well as metabolism-related genes, including *BSCL2* and *ALDH18A1*. *ALDH18A1* variants, which showed a very high frequency in our data, are considered to cause HSP through mitochondrial ornithine deficiency [47,48], which could be an important therapeutic target in Korean patients. These results indicate that in Korean patients with HSP, the major causative gene is *SPAST*, with the other HSP genes being rare and diverse. Furthermore, CN patients showed two
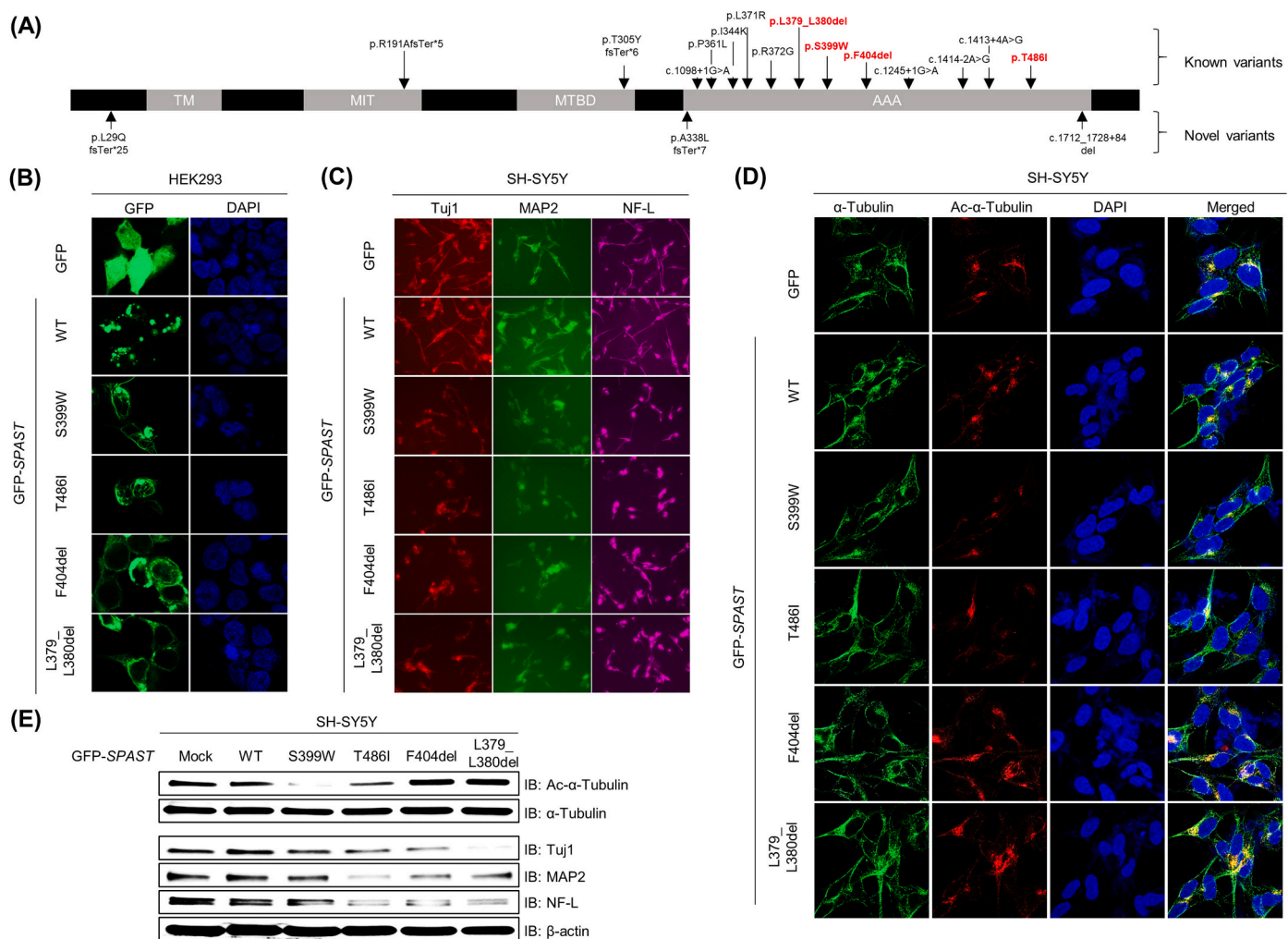
Fig. 6. *SPAST* variants that dysregulate neurite outgrowth and microtubule stability. (A) Summary of *SPAST* mutations identified in our study. Red represents four *SPAST* variants of unknown clinical significance or having no functional data in the ClinVar database. (B) HEK293 cells were transfected with the indicated wild-type and mutant vectors (S399W, T486I, L379_L380del, and F404del). There was the localization of SPAST-WT and SPAST-mutants in cells expressing GFP-SPAST. (C-E) Human SH-SY5Y neuroblastoma cells transfected with the indicated vectors were allowed to differentiate with retinoic acid for 72 h. The cells were stained using antibodies against neuron-specific markers MAP2, NF-L, and Tuj1 (C), as well as α-tubulin and its acetylated form (D). DAPI (blue) was used for nuclear staining. (E) The expression levels of indicated proteins were analyzed using Western blotting. We used β-actin and α-tubulin as a loading control.

major causative HSP genes, including *SPAST* and *SPG11*, which are consistent with previous findings [49–51], mainly with Ins/Del/Dup-type mutations. Compared with other ethnic groups, HU patients have a higher frequency of *SPG7* variants. These findings suggest that these genetic differences represent ethnic-specific loci of HSP causal variations; moreover, the wide range of mutation frequency across ethnic groups could facilitate HSP elucidation.

Overall, this large-scale study on well-defined Korean patients showed a highly accurate and expanded molecular characterization for HSP candidate genes, which expands the genetic spectrum of Korean HSPs. Furthermore, we experimentally validated the pathogenicity of several unknown functional variants in *SPAST* and *REEP1* genes. Additionally, we confirmed the diversity of known HSP among five ethnic groups, which indicated ethnic differences in the HSP genotypic features. Our findings could contribute toward precise diagnosis and management of patients with HSP who remain undiagnosed and untreated given their rarity.

Data availability

All the data that support the findings of this study are available from the corresponding author upon reasonable request.

Author's contributions

JOY, J-YY, and N-SK conceived and designed the study. JOY, J-YY, SY, DH, and N-SK drafted the manuscript. JOY, J-YY, JKC, BJK, and N-SK performed breakpoints verification. JOY, J-YY, J-JL, SY, DH, and E-JW participated in the data analysis. DHS, SY, J-JL, SYJ, S-JJ, JKC, and BJK reviewed and edited the manuscript and contributed to the discussions. DHS, JMS, JWC, J-HJ, and BJK participated in clinical data collection. N-SK supervised the study. All authors reviewed and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.10.014.

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