



RESEARCH ARTICLE

Biallelic *DDHD2* mutations in patients with adult-onset complex hereditary spastic paraplegiaYing-Tsen Chou^{1,*}, Shao-Lun Hsu^{1,2,*}, Yu-Shuen Tsai³, Yi-Jiun Lu⁴, Kai-Wei Yu⁵, Hsiu-Mei Wu⁵, Yi-Chu Liao^{1,2,6}  & Yi-Chung Lee^{1,2,6,7,8} ¹Department of Neurology, Taipei Veterans General Hospital, Taipei, Taiwan²Department of Neurology, National Yang Ming Chiao Tung University School of Medicine, Taipei, Taiwan³Center for Systems and Synthetic Biology, National Yang Ming Chiao Tung University, Taipei, Taiwan⁴Department of Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, Taipei, Taiwan⁵Department of Radiology, Taipei Veterans General Hospital, Taipei, Taiwan⁶Brain Research Center, National Yang Ming Chiao Tung University, Taipei, Taiwan⁷Department of Biological Science and Technology, College of Biological Science and Technology, National Yang Ming Chiao Tung University, Hsinchu, Taiwan⁸Center for Intelligent Drug Systems and Smart Bio-devices (IDS²B), National Yang Ming Chiao Tung University, Hsinchu, Taiwan**Correspondence**

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Abstract

Objective: Hereditary spastic paraplegias (HSPs) are a group of inherited neurodegenerative disorders characterized by slowly progressive lower limb spasticity and weakness. HSP type 54 (SPG54) is autosomal recessively inherited and caused by mutations in the *DDHD2* gene. This study investigated the clinical characteristics and molecular features of *DDHD2* mutations in a cohort of Taiwanese patients with HSP. **Methods:** Mutational analysis of *DDHD2* was performed for 242 unrelated Taiwanese patients with HSP. The clinical, neuroimaging, and genetic features of the patients with biallelic *DDHD2* mutations were characterized. A cell-based study was performed to assess the effects of the *DDHD2* mutations on protein expression. **Results:** SPG54 was diagnosed in three patients. Among them, two patients carried compound heterozygous *DDHD2* mutations, p.[R112Q]; [Y606*] and p.[R112Q]; [p.D660H], and the other one was homozygous for the *DDHD2* p.R112Q mutation. *DDHD2* p.Y606* is a novel mutation, whereas *DDHD2* p.D660H and p.R112Q have been reported in the literature. All three patients manifested adult onset complex HSP with additional cerebellar ataxia, polyneuropathy, or cognitive impairment. Brain proton magnetic resonance spectroscopy revealed an abnormal lipid peak in thalamus of all three patients. In vitro studies demonstrated that all the three *DDHD2* mutations were associated with a considerably lower *DDHD2* protein level. **Interpretation:** SPG54 was detected in approximately 1.2% (3 of 242) of the Taiwanese HSP cohort. This study expands the known mutational spectrum of *DDHD2*, provides molecular evidence of the pathogenicity of the *DDHD2* mutations, and underlines the importance of considering SPG54 as a potential diagnosis of adult-onset HSP.

Introduction

Hereditary spastic paraplegias (HSPs) are a group of inherited neurodegenerative disorders mainly presenting with progressive lower extremity spasticity and weakness.^{1,2} Clinically, HSPs are classified into pure or complex forms. Pure HSPs are characterized by progressive lower limb spastic weakness, hypertonic urinary bladder, and mild

diminution of vibrational sensation in the lower limbs, whereas complex HSPs have additional neurological or non-neurological manifestations, such as ataxia, seizure, cognitive impairment, and peripheral neuropathy.^{3,4} Currently, more than 89 genes or genetic loci have been implicated in HSPs and the inheritance pattern of HSP subtypes can be autosomal dominant (AD), autosomal recessive (AR), or X-linked inherited.^{3–6}

Spastic paraplegia type 54 (SPG54; MIM# 615033) is a rare AR complex HSP caused by biallelic mutations in the *DDHD2* gene. Thirty-four variants of *DDHD2* mutations have been reported in SPG54 patients.^{7–14} Most of the variants result in loss-of-function of the protein product (pLoF variants), including nonsense or frameshift truncating mutations, and a minor group of variants are missense mutations, including p.Y56C, p.W103R, p.R112Q, p.T186M, p.G197R, p.V220F, p.R242C, p.R242H, p.P269L, p.P339S, p.Q487H, p.F502S, p.G510E, p.D660H, p.C683S, and p.Y699C.^{7–14} Among these mutations, only p.R287* and p.D660H have been identified in more than two families.^{8,15,16} Most SPG54 patients have early-onset disease, with symptoms presenting in the first decade of life.^{10,17,18} Adult-onset SPG54 has only been reported in four patients from three families; three of these patients presented with spastic-ataxia syndrome.^{7,10} The common neuroimaging features of SPG54 include a thin corpus callosum (TCC), white matter hyperintensity (WMH) in magnetic resonance imaging (MRI), and lipid accumulation in the thalami, and basal ganglia detected by proton magnetic resonance spectroscopy (¹H-MRS).^{10,15,17,19}

The protein encoded by *DDHD2* is DDHD-domain-containing 2 protein (DDHD2), which belongs to the intracellular phospholipase A1 (PLA1) family and hydrolyzes fatty acid substrates from the *sn*-1 position of phospholipids.^{5,20} DDHD2 also exhibits triglyceride hydrolase activities, which modulate triglyceride metabolism and lipid accumulation in the brain.^{21–23} DDHD2 is ubiquitously located and expressed in subcellular membrane compartments in the perinuclear area or proximal to the cis-Golgi apparatus, indicating potential regulation of membrane trafficking.^{22,24} Although molecular mechanisms of SPG54 remained unclear, previous functional studies demonstrated markedly decreased PLA1 activities in cells expressing *DDHD2* W103R, V220F, and D660H mutant proteins,⁷ suggesting that *DDHD2* mutations may cause SPG54 through a loss-of-function effect.

In this study, we investigated the mutational spectrum and clinical phenotypes of SPG54 in a cohort of 242 unrelated Taiwanese patients with HSP. The neuropsychological, electrophysiological, and neuroimaging features of the patients carrying biallelic *DDHD2* mutations were investigated. We further assessed the effects of the identified *DDHD2* mutations on protein expression with *in vitro* study.

Methods

Study subjects

A continuous series of 242 unrelated patients with a clinical diagnosis of HSP were recruited at the Department of

Neurology, Taipei Veterans General Hospital from January 1998 to January 2022. The patients had either (1) pure spastic paraplegia, (2) spastic quadriplegia with earlier and more severe involvement of lower limbs or (3) spastic paraplegia as an early and prominent sign of a neurodegenerative disease involving multiple parts of the nervous system. Other disease etiologies were excluded through brain and spine imaging studies and basic biochemical tests.^{25–27} Among the 242 patients, 133 were men and 109 were women, and 95 patients (39%) had a family history of HSP phenotype, including 72 AD families, 19 AR families, and 4 families with X-linked inheritance. The other 147 (61%) were apparently sporadic cases. The average age of disease onset was 29.2 ± 17.7 years (range: 0–69 years). All the participants were Taiwanese of Han Chinese ethnicity. Peripheral blood samples were collected from participants after their written informed consent was obtained. The protocols of this study were approved by the Institutional Review Board of Taipei Veterans General Hospital.

Mutational analysis

Mutational analysis of *DDHD2* was performed with a targeted resequencing panel covering 76 confirmed HSP disease genes as well as 57 genes associated with diseases manifesting HSP-like phenotype on an Illumina HiSeq2500 platform (Table S1). Sequenced read alignment and variant calling were performed with the reference Human Genome version 38 (hg38/GRCh38). The *DDHD2* pathogenic variants were verified with Sanger sequencing and named according to the reference *DDHD2* sequence (NM_015214.3). The pathogenicity of the novel *DDHD2* mutation was assessed according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) guidelines.²⁸

Clinical, neuropsychological, and electrophysiological evaluations and imaging studies

The probands and their families received complete neurological examinations. Disease severity was evaluated with Spastic Paraplegia Rating Scale (SPRS)²⁷ and SPATAX-EUROSPA disability score.²⁹ SPRS is a validated HSP-specific severity scale with 13 items and is used to measure functional impairment of walking ability, muscle strength, spasticity, pain, and urinary function. Each item was scored from 0 (full function) to 4 (most severe impairment). The SPATAX-EUROSPA disability score grades functional impairment as 0 (no functional handicap), 1 (showing signs at examination), 2 (able to run,

walking unlimited), 3 (unable to run, limited walking without aid), 4 (walking with one stick), 5 (walking with two sticks), 6 (requiring wheelchair), and 7 (confined to bed). The Scale for Assessment and Rating of Ataxia (SARA) were also used to quantify disease severity in patients with cerebellar manifestations.³⁰ Mini-Mental State Examination (MMSE) was performed to assess cognitive function.³¹ Nerve conduction studies (NCSs) were conducted by standard techniques utilizing a Medelec MS25 electromyograph (Mistro, Surrey, UK) with surface electrode stimulations and recordings.³² Brain and spine MRI were performed with 3-Tesla MR system (Signa EXCITE, GE Medical Systems, Milwaukee, WI, USA). T1-weighted images (T1WI), T2-weighted images (T2WI) and ¹H-MRS were reviewed.

Expression plasmids, cell culture, and transfection

A wild-type (WT) human *DDHD2* expression plasmid with FLAG-tagged in the C-terminal region (backbone: pcDNA3.1⁺/c-(K)-DYK; clone ID: OHu23306) was purchased from GenScript (Piscataway, NJ, USA). The mutations, including c.335G>A (p.R112Q), c.1818C>A (p.Y606*), and c.1978G>C (p.D660H), were incorporated into the WT construct, respectively, by PCR-based site-directed mutagenesis method using Pfu Turbo DNA polymerase (Agilent, Santa Clara, CA, USA). Given that the p.Y606* mutation may lead to premature truncation of the protein product, the C-terminal FLAG-tag (DYKDDDDK) of all *DDHD2* constructs (WT and mutants) was moved to the N-terminal using Q5 Site-Directed Mutagenesis Kit according to the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA).

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum at 37°C under 5% CO₂. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Reverse-transcription polymerase chain reaction (RT-PCR)

The total RNA of the transfected cells was harvested 24 h after transfection using Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). The cDNA synthesis was conducted using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The *DDHD2* mRNA expression was normalized against *GAPDH* mRNA expression. Data analysis and plots were generated using Prism (GraphPad Software, San Diego, CA, USA).

Analysis of DDHD2 protein levels and cycloheximide (CHX)-chase assays

Twenty-four hours post-transfection, cells were harvested and analyzed with western blotting. The steady-state *DDHD2* protein levels were analyzed using anti-FLAG antibody (#8146, Cell Signaling Technology, Danvers, MA, USA). β -Actin was used as a loading control and detected with anti- β -actin antibody (#8226; Abcam, Cambridge, UK). To determine the stability of the WT and mutant *DDHD2* proteins, CHX-chase assays were conducted. At 24 h post-transfection, the transfected cells were treated with 100 mg/ μ l cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) for 0–8 h and harvested at the indicated time points. Cell lysates were prepared and then subjected to western blotting with the anti-FLAG antibody. Actin was used as loading control. The ratios of *DDHD2* to actin were calculated densitometrically. Data analysis and plots were generated using Prism (GraphPad Software, San Diego, CA, USA).

Results

Identification of the DDHD2 mutations

Three patients in the HSP cohort carried biallelic *DDHD2* mutations, including two patients with compound heterozygous mutations, p.[R112Q];[Y606*] (c.[335G>A]; [1818C>A]) and p.[R112Q];[D660H] (c.[335G>A]; [1978G>C]), and one with homozygous p.R112Q (c.335G>A) mutation (Fig. 1A–C). Among these mutations, *DDHD2* p.Y606* is novel, whereas p.R112Q and p.D660H have been reported to be associated with SPG54 in previous studies.⁹ The p.Y606* mutation is a nonsense mutation that may disrupt the DDHD domain and lead to *DDHD2* protein truncation (Fig. 1D). This mutation was absent in the Genome Aggregation Database (gnomAD v2.1.1; <http://gnomad.broadinstitute.org/>), as well as the 1517 ethnically matched control genomes from the Taiwan Biobank database (<http://www.twbiobank.org.tw/>). According to the ACMG-AMP guidelines,²⁸ the *DDHD2* p.Y606* mutation was predicted to be a null variant (PVS1 criterion); absent in population databases (PM2), capable of disrupting the DDHD domain (PM1); capable of shortening the length of *DDHD2* protein (PM4); detected in *trans* with a known pathogenic mutation, p.R112Q (PM3), and identified in a patient with HSP (PP4). Thus, *DDHD2* p.Y606* is classified as a pathogenic variant.

Clinical information and other evaluations

The clinical information of patients with SPG54 was summarized in Table 1. The proband of family A (A-II-2)

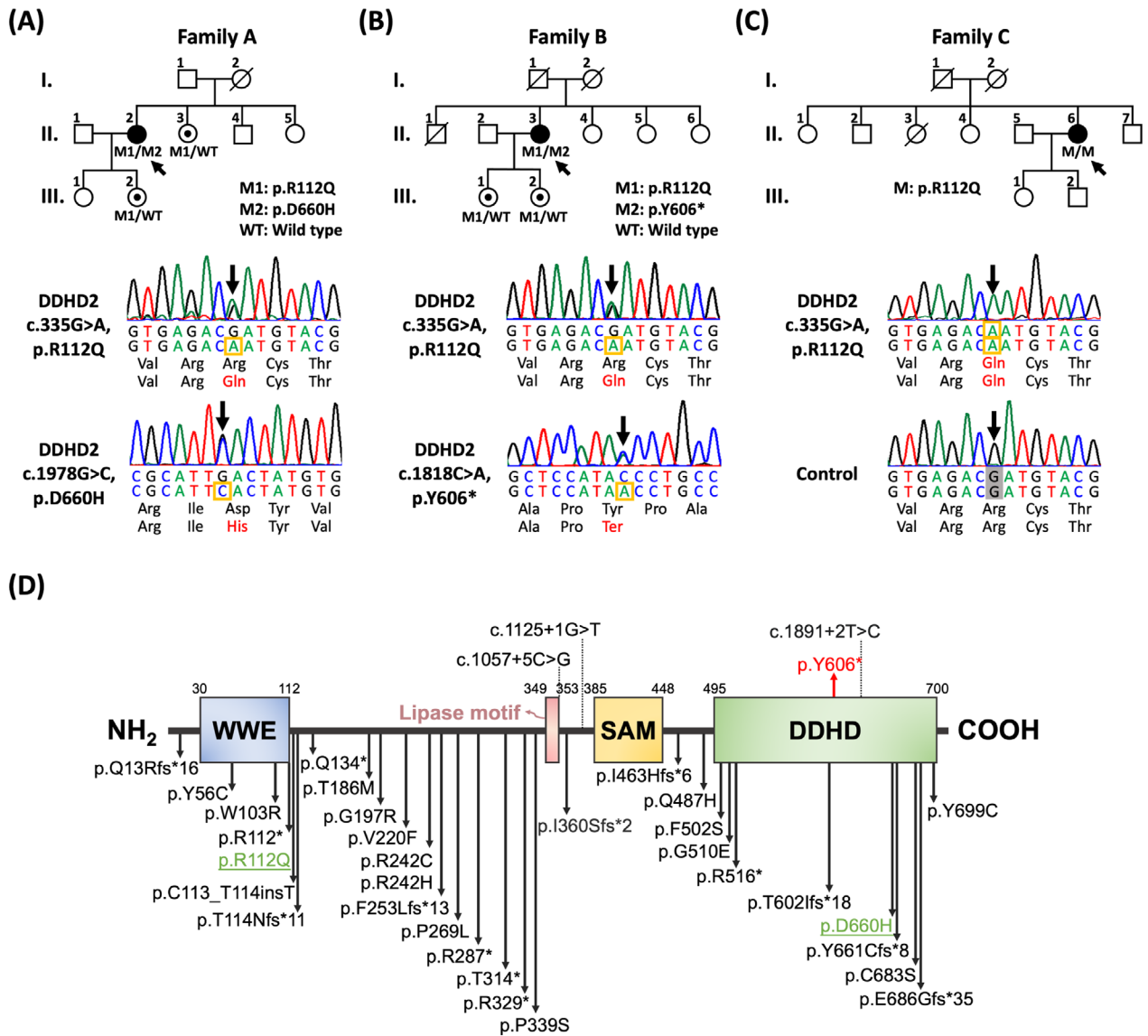


Figure 1. Genetic analysis of the patients with spastic paraplegia type 54 (SPG54). (A–C) Pedigrees of the three SPG54 families and the Sanger sequencing traces of *DDHD2* mutations including *DDHD2* c.[335G>A];[1978G>C] (p.[R112Q];[D660H]) and *DDHD2* c.[335G>A];[1818C>A] (p.[R112Q];[Y606*]) in compound heterozygous form and *DDHD2* c.335G>A (p.R112Q) in homozygous form in families A, B, and C, respectively. The probands are indicated by arrows. The “M” represents a mutant *DDHD2* allele, and the “W” refer to a wild-type allele. The squares and circles denote for males and females, separately. The filled and open symbols represent affected and unaffected members, respectively. Dotted symbols indicate obligate carriers. The altered amino acid residues are labeled in red. (D) Scheme of the *DDHD2* protein and its domains (WWE, lipase, sterile-alpha-motif (SAM), and DDHD), labeling with SPG54-related mutations identified in the literatures (marked in black) and the present study (the novel mutation marked in red, and the recurred mutations marked in green).

carrying the *DDHD2* p.[R112Q];[D660H] mutation was a 61-year-old woman born to non-consanguineous parents with no family history of neurological diseases. Her motor developmental milestones in childhood were normal, but she had insidious motor function regression and unsteady gait since age 35, which indicated pyramidal and cerebellar manifestations. The patient was able to stand

and walk independently until age 55. Neurological examination at age 61 revealed lower limb spasticity, mild weakness of hip flexors, and increased deep tendon reflexes (DTRs) of biceps and knee but decreased DTRs of ankle. She also had bilateral extensor plantar responses, and prominent limb, and truncal ataxia. Impaired sensation of all modalities over distal limbs were also noted,

Table 1. Clinical characteristics of Taiwanese SPG54 patients.

Patients	A-II-2	B-II-3	C-II-6
Genetic variants	c.[335G>A]; [1978G>C]	c.[335G>A]; [1818C>A]	c.[335G>A]; [c.335G>A]
Protein	p.[R112Q];[D660H]	p.[R112Q];[p.Y606*]	p.[R112Q];[p.R112Q]
Gender	Female	Female	Female
Age at onset (years)	35	45	50
Disease duration (years)	26	23	12
SPRS score (decline rate)	12 (0.46/year)	32 (1.39/year)	22 (1.83/year)
SARS score (decline rate)	11 (0.42/year)	12 (0.52/year)	9 (0.75/year)
Disability score (0–7) ^a	3	5	5
Spasticity (Knee extension)	+	+	+
Muscle strength (Hip flexors) ^b	4	4	4
DTR ^c			
Biceps	+++	+++	++
Knee	+++	+++	+++
Ankle	+	++	+
Babinski sign	+	+	+
Sensory deficits			
Surface	+	–	+
Position/vibration	+	+	+
Urinary symptoms	–	+	–
Cerebellum ataxia	+	–	–
Cognitive impairment	+	+	–
Peripheral neuropathy	+	–	+

–, absence; +, presence; DTR, deep tendon reflex; SARA, Scale for Assessment and Rating of Ataxia; SPRS, Spastic Paraplegia Rating Scale.

^aThe disability stage 3 means “unable to run, limited walking without aid” and stage 5 means “walking with two sticks.”

^bMedical Research Council (MRC) scale for muscle strength: 0–5.

^cMRC scale for tendon reflex: – to ++++.

indicating a length-dependent peripheral neuropathy. Her SPRS score was 12 (Table S2) and her SARA score was 11. The patient could walk without aid for a short distance (disability score: 3; Video S1). Her MMSE score at age 61 was 19 out of 30, which indicated cognitive impairment. The NCS showed axonal sensorimotor polyneuropathy (Table S3). Brain and spine MRIs were normal. However, her brain ¹H-MRS revealed an abnormal lipid peak in bilateral thalami (Fig. 2A). The patient's sister and daughter were heterozygous to *DDHD2* p.R112Q mutation and presented normal findings in neurological examinations at age 59 and 38, respectively.

The proband of family B (B-II-3), who carried *DDHD2* p.[R112Q];[Y606*] mutation, was a 68-year-old woman born to non-consanguineous parents without family history of neurological diseases. Her early motor steps were normal. Initial symptoms included lower extremity stiffness and frequent falls beginning at age 45. Her motor function deteriorated gradually, and from age 60 onward, she only could walk fewer than 10 steps at a time. She also experienced urinary urgency and occasional

incontinence. A neurological examination at age 68 revealed lower limb spasticity, mild weakness of hip flexors and knee flexors, general hyperreflexia and bilateral extensor plantar responses. Impaired vibration sensation of the distal lower limbs was also present. Her SPRS score was 32 (Table S2). The patient required two assistive canes to walk (disability score: 5; Video S1). Her MMSE score was 23 out of 30, indicating cognitive impairment. Her NCS (Table S3) and conventional brain and spine MRIs were normal. However, her brain ¹H-MRS demonstrated an abnormal lipid peak in bilateral thalami (Fig. 2B). Her two daughters, who carried heterozygous *DDHD2* p.R112Q mutation, both presented healthy with normal findings in neurological examinations at age 46 and 43.

The proband of family C (C-II-6) was a 62-year-old woman carrying homozygous *DDHD2* p.R112Q mutation. She was from a non-consanguineous family with normal early motor development and began to have gait disturbance at age 50. Then, her motor ability worsened continuously and she could walk only with bilateral assistance

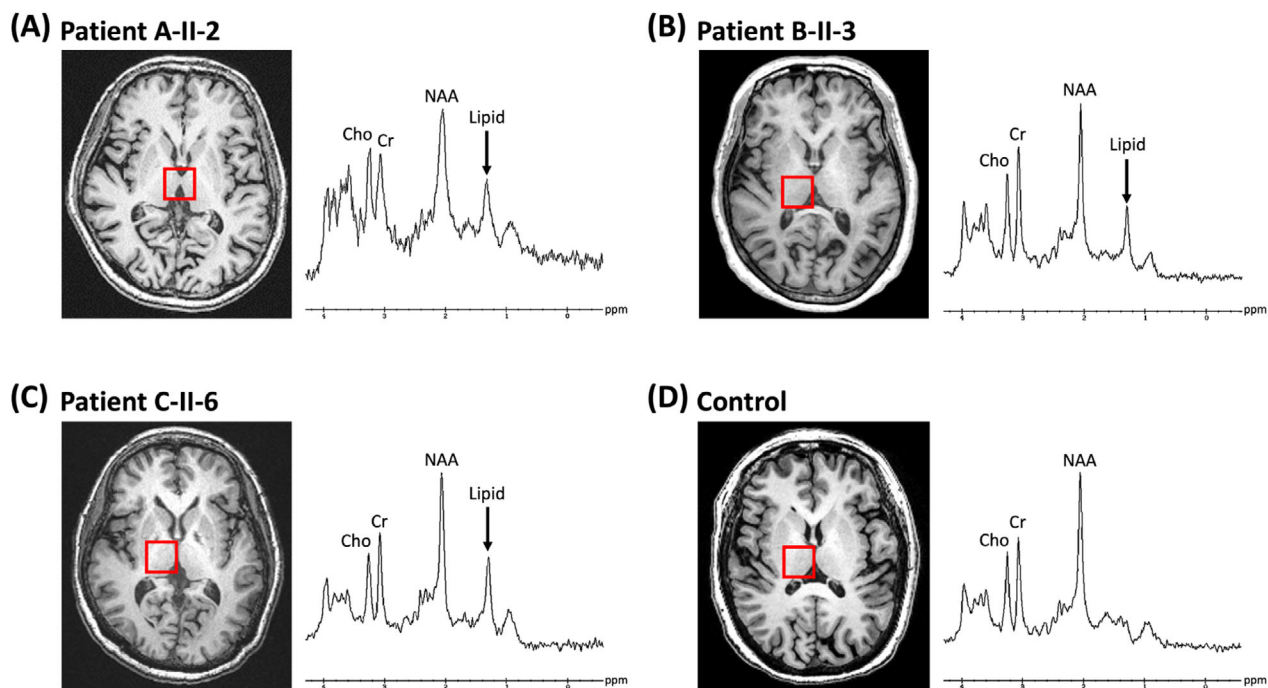


Figure 2. Proton magnetic resonance spectroscopy obtained from the patient A-II-2 (A), patient B-II-3 (B), patient C-II-6 (C) and healthy control (D) at a magnetic field of 3-Tesla (echo time 35 ms). The arrows indicate the pathologic lipid peak at 1.3 ppm detected at bilateral thalami. Cho, choline; Cr, creatine; NAA, N-acetylaspartate.

at age 59. A neurological examination at age 62 revealed lower limb spasticity, mild weakness of hip flexors, increased DTRs of knee but decreased in ankle jerks, and bilateral extensor plantar responses. Impaired sensation of all modalities over the distal lower limbs indicated a length-dependent peripheral neuropathy. Her SPRS score was 22 (Table S2). The patient required two assistive canes for walking (disability score: 5; Video S1). The NCS showed axonal sensorimotor polyneuropathy (Table S3). Brain and spine MRIs were normal. The cerebral ^1H -MRS also revealed an abnormal lipid peak in bilateral thalami (Fig. 2C).

DDHD2 mutations decreased steady-state protein levels and protein stability

To investigate the effects of the three *DDHD2* mutations, we examined the mRNA and protein expression using HEK293T cells transfected with FLAG-tagged WT or mutated forms of *DDHD2* constructs. An RT-PCR analysis showed that *DDHD2* p.R112Q, p.Y606*, and p.D660H mutations did not change the mRNA expression of *DDHD2* (Fig. 3A). However, a western blot analysis showed a dramatic decrease in protein levels of the R112Q and D660H mutants at 24 h post-transfection (Fig. 3B and C). As expected, the p.Y606* mutant

construct produced truncated protein products, which level was only minimal at steady-state compared to that of *DDHD2*-WT (Fig. 3B and C). Because the reduced levels of mutant *DDHD2* protein were independent from mRNA levels, we further tested whether the stabilities of mutant proteins changed. The cycloheximide chase assay showed that p.R112Q and p.D660H mutations had significantly accelerated the degradation of *DDHD2* proteins (Fig. 3D and E). Similar results were found in C-terminal FLAG-tagged *DDHD2* proteins (Figure S1). The p.Y606* was not assessed because the mutant protein level was too low.

Discussion

We screened 242 unrelated Taiwanese patients with HSP and identified three SPG54 patients carrying biallelic *DDHD2* mutations. Among them, two patients carried compound heterozygous *DDHD2* mutations, p.[R112Q];[D660H] and p.[R112Q];[Y606*], and one patient was homozygous for the *DDHD2* p.R112Q mutation. Among these mutations, *DDHD2* Y606* was novel. There are several interesting findings. First, *DDHD2* mutations accounted for 1.2% (3 out of 242) of all HSP patients and 2% (3 out of 147) of patients with AR or sporadic HSP in the Taiwanese cohort. Second, SPG54 in our

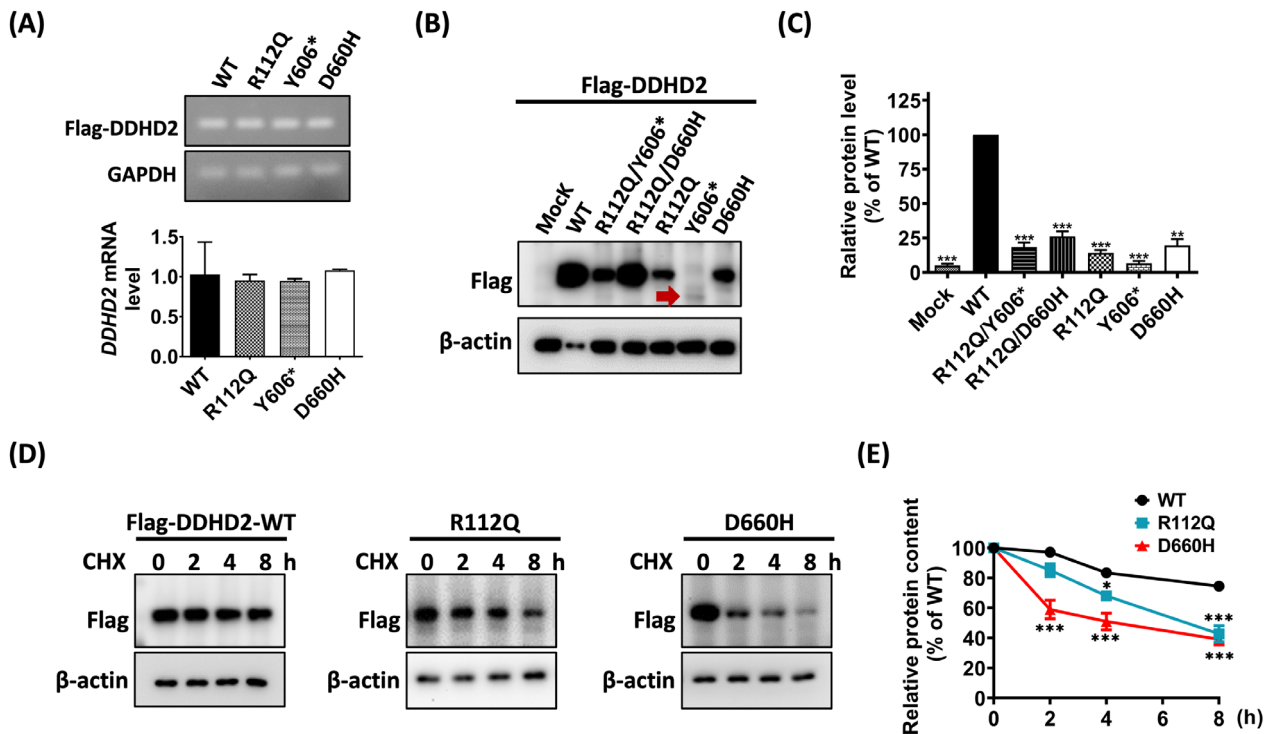


Figure 3. In vitro functional study of the identified *DDHD2* variants in HEK293T cells. (A) Representative reverse-transcription polymerase chain reaction analysis of *DDHD2* mRNA levels in HEK293T cells transfected with different *DDHD2* constructs. *GAPDH* mRNA was used as a loading control. Densitometric quantification is shown below. The error bars indicate standard error of the mean (SEM) from three independent experiments. (B) Representative western blot analysis for steady-state expression of *DDHD2* proteins in HEK293T cells transfected with different *DDHD2* constructs. The band of the truncated *DDHD2*-Y606* protein was labeled by a red arrow. β -actin was used as a loading control. (C) Relative *DDHD2* mutant protein expression at steady-state was normalized to β -actin and calculated in reference to *DDHD2*-WT/ β -actin. Data were obtained from four independent experiments. Statistical analysis was performed by one-way ANOVA followed by *post hoc* analysis ($n = 4$). (D) HEK293 cells were transfected with either *DDHD2*-WT, R112Q, or D660H constructs for 24 h and subsequently subjected to cycloheximide (CHX)-chase assays. Data were obtained from three independent experiments. Representative western blot analysis is shown. (E) The quantified results of the CHX-chase assays. Two-way ANOVA and *post hoc* Tukey tests were performed at every time point ($n = 3$). All data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for *DDHD2* mutants versus Wild-type groups.

study presented as adult-onset complex HSP with additional manifestations including ataxia, neuropathy, or cognitive impairment. Third, ^1H -MRS showing abnormal lipid peak in thalamus was a common and distinctive neuroradiological characteristic of SPG54. Finally, in vitro studies showed that three *DDHD2* mutations (p.R112Q, p.Y606*, and p.D660H) resulted in severely decreased steady-state protein levels, which were independent of the mRNA levels but may be associated with accelerated degradation of the mutant proteins.

SPG54 is an uncommon HSP subtype. An Italian study investigated 239 unrelated patients with HSP and found that SPG54 accounted for 1.7% of overall cases and 2.4% of the patients with AR or apparently sporadic HSP.³³ In a large cohort of French people with HSP, 7 out of 1550 index patients carried biallelic *DDHD2* mutations, which represented 0.45% (7 out of 1550) of all patients with HSP and 3.8% (7 out of 186) of patients with AR-HSP.¹²

In a Chinese cohort with 99 HSP patients and the Japan Spastic Paraplegia Research Consortium (JASPAC) cohort with 383 HSP patients, both studies reported that fewer than 1% of their respective patients carried *DDHD2* mutations.^{9,34} However, in pediatric-onset HSP cohorts, SPG54 was responsible for approximately 2% of HSP cases, which indicated that SPG54 more frequently caused HSP symptoms in early childhood.^{13,14,35} Concordant to the results observed in other ethnic groups, SPG54 accounted for only 1.2% (3 out of 242) of all patients with HSP and 2% (3 out of 147) of patients with AR or apparently sporadic HSP in our Taiwanese cohort. These findings suggest that SPG54 is a rare HSP subtype and should be considered only in patients with AR or apparently sporadic HSP.

SPG54 typically presents with pediatric-onset HSP. Previous reports have indicated that over 90% of SPG54 patients had congenital- or infantile-onset delay in motor

and cognitive milestones, followed by lower limb predominant spastic weakness. Additional features included cerebellar ataxia, abnormal ocular movements, strabismus, optic nerve hypoplasia, color vision deficiency, and short stature.^{10,15,17,18,36} Adult-onset SPG54 is relatively rare in the literatures. Only four adult-onset cases have been reported. Three of the patients presented with ataxic-spastic syndrome since 40s and none of the patients had cognitive impairment.^{7,10} Surprisingly, we found three adult-onset SPG54 patients and they all demonstrated complex HSP phenotypes with variable extra characteristics. All three patients manifested lower limb spasticity beginning at middle age. Patient A-II-2 had prominent cerebellar ataxia, axonal sensorimotor polyneuropathy and cognitive impairment. Patients B-II-3 and C-II-6 mainly demonstrated severe spasticity accompanied with cognitive impairment or polyneuropathy. These findings suggest that both infantile-onset and adult-onset SPG54 may present with complex HSP. There was no clear association between the onset age and the core features of neurological defects.

Among our three patients with SPG54 and the aforementioned four adult-onset SPG54 cases,^{7,10} more than half of the adult-onset HSP cases (4 out of 7) exhibited additional symptoms such as cerebellar ataxia and polyneuropathy. These findings suggest that adult-onset SPG54 usually manifests as ataxia-spasticity spectrum disease (ASSD), which is a large group of inherited neurodegenerative diseases characterized by spasticity and ataxia.³⁷ The differential diagnoses of ASSD include autosomal recessive ataxia of Charlevoix-Saguenay, late-onset Friedreich ataxia, adult-onset Alexander disease, spinocerebellar ataxia types 3, 7, 17, 23, 28, 40, and 42, Spastic ataxia types 1–9, and some AR-HSP (SPG5, SPG7, SPG11, SPG15, SPG20, and SPG21).³⁸ Our finding indicates that SPG54 is also a member of ASSD and should be considered in patients with overlapping spasticity and ataxia of unknown cause, especially in those with adulthood onset disease, AR or apparently sporadic inheritance, and superimposed polyneuropathy.

MRI and ¹H-MRS are useful diagnostic tools for SPG54. In MRI studies of SPG54, TCC and WMH both were detected in over 70% of SPG54 patients.^{10,17} However, TCC and WMH are not features specific to SPG54, and are also commonly found in patients with other AR-HSP including SPG7, SPG11, SPG15, SPG18, SPG21, SPG35, SPG46, SPG47, SPG49, SPG50, and SPG63.^{17,19,39} An abnormal lipid peak of 1.3 ppm identified in basal ganglia and thalami with ¹H-MRS was regarded as an important imaging marker for SPG54.^{15,40} The mechanism underlying abnormal lipid peak may be related to the function of DDHD2 protein, which is both a phospholipase and a triglyceride lipase in the central nerve

system. A recent study showed that depletion of *Ddhd2* in mice resulted in massive accumulation of triglyceride and lipid droplets in the brain and spinal cord.²¹ It was still unclear whether other HSP subtypes (including SPG5, SPG28, SPG31, SPG38, SPG39, SPG46, and SPG56) with disease genes related to lipid metabolism could also exhibit abnormal lipid peaks in ¹H-MRS.^{5,23} In our study, the brain and spine MRIs of three adult-onset SPG54 patients were grossly normal, despite the extended duration of the disease (26, 23, and 12 years). However, the ¹H-MRS of all our three SPG54 patients demonstrated an abnormal lipid peak in the bilateral thalami (Fig. 2). This suggested that ¹H-MRS is more sensitive than conventional MRI in detecting neuroradiological abnormalities in SPG54.

We provided in vitro evidence supporting the pathogenicity of *DDHD2* p.R112Q, p.Y606*, and p.D660H mutations. SPG54 is an AR disorder more frequently caused by nonsense or frameshift truncating mutations. Markedly decreased PLA1 activities of *DDHD2* mutant proteins have been demonstrated in functional studies of many pathogenic *DDHD2* missense variants, including p.W103R, p.V220F, and p.D660H.⁷ These studies have indicated that SPG54 is caused by the loss-of-function effect of *DDHD2* mutations. Through cell transfection studies, we demonstrated that the p.R112Q, p.Y606*, and p.D660H mutations led to vastly decreased *DDHD2* protein levels (Fig. 3B and C). Moreover, we showed that *DDHD2*-R112Q and *DDHD2*-D660H mutant proteins had faster protein degradation than *DDHD2*-WT (Fig. 3D and E). These findings suggest that these *DDHD2* mutations may lead to SPG54 with a loss-of-function effect through compromising protein stability and greatly decreasing *DDHD2* protein expression levels.

In conclusion, SPG54 is a differential diagnosis of AR-HSP or sporadic HSP in Taiwan, and *DDHD2* mutations account for 2% of AR or sporadic HSP cases. *DDHD2* Y606* is a novel pathogenic mutation of SPG54. Our study broadens the spectrum of *DDHD2* mutations, provides molecular evidence supporting the pathogenicity of the *DDHD2* mutations, and highlights the potential for SPG54 as an diagnosis of adult-onset HSP.

Author Contributions

Ying-Tsen Chou, Shao-Lun Hsu, Yi-Chu Liao, Yi-Chung Lee contributed to conception and design of the study. Ying-Tsen Chou, Shao-Lun Hsu, Yu-Shuen Tsai, Yi-Jiun Lu, Kai-Wei Yu, Hsiu-Mei Wu contributed to acquisition and analysis of data. Ying-Tsen Chou, Shao-Lun Hsu, Yi-Chu Liao, Yi-Chung Lee contributed to drafting of the manuscript.

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Conflict of Interest

The authors declared no conflict of interests.

Data Availability Statement

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

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Supporting Information

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

Table S1. The gene list of the targeted resequencing panel for HSP.

Table S2. The score of Spastic Paraplegia Rating Scale in Taiwanese SPG54 patients.

Table S3. Nerve conduction studies of the Taiwanese patients with spastic paraplegia type 54.

Figure S1. Cycloheximide-chase assay of the C-terminal FLAG-tagged DDHD2 variants in HEK293T cells. (A) HEK293 cells were transfected with vectors carrying either C-terminal FLAG-tagged DDHD2-WT, R112Q, or D660H for 24 h and subsequently subjected to cycloheximide (CHX)-chase assays for indicative times. Representative western blot analysis is shown. β -Actin was used as a loading control. (B) The quantified results of the CHX-chase assays from three independent experiments. One-way ANOVA and post hoc Dunnett's tests were performed at every time point. All data are presented as mean \pm SEM. * $P < 0.05$ and ** $P < 0.01$ for DDHD2 mutants versus wild-type groups.

Video S1. The videos of gait of the SPG54 patients, A-II-2, B-II-3, and C-II-6 at age 61, 68, and 62 years, respectively.