The Genetics of Hereditary Spastic Paraplegia

Mehrdad Asghari Estiar, MSc

Department of Human Genetics, Faculty of Medicine and Health Sciences,

McGill University, Montreal, Canada

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Table of Contents

Abstract

Background: Hereditary spastic paraplegias (HSPs) are a group of rare monogenic Mendelian neurodegenerative disorders characterized by lower limb spasticity with/without additional symptoms. Thus far, 86 causative genes or loci have been described for HSP with the emergence of next-generation sequencing. However, more than half of the HSP patients remain genetically unsolved even with the use of exome sequencing (ES). There are some potential explanations to account for a large proportion of genetically undiagnosed patients. This includes the limitation of ES to detect certain variations, the mutation occurrences within new or non-HSP genes, and the currently oversimplified concept of HSP's genetic inheritance.

Objectives: The general objective of this study was to better understand HSP and improve its genetic diagnostic yield. **Aim 1)** Identify disease-causing variants in genes involved in HSP in Canada. **Aim 2)** Identify disease-causing variants in potentially new and non-HSP genes. **Aim 3)** Studying evidence for complex inheritance in common HSP subtypes.

Methods: A total of 608 individuals from 375 HSP families have been recruited across Canada. HSP-gene panel sequencing was performed on 435 individuals. Collectively, 422 individuals from 294 families underwent ES. ES data of 580 individuals were also used for analysis as a control group. Among unsolved cases, 52 patients went through genome sequencing (GS). The suspicious variants were validated by Sanger sequencing. Multiple bioinformatic and *in silico* tools were applied to detect the disease-causing variants. For detection of CNVs in ES data, ExomeDepth tool was applied, and Multiplex ligation-dependent probe amplification was used for validation.

Results: We identified 256 out of 375 index cases with potentially pathogenic variants across 33 known HSP genes, 19 genes implicated in overlapping syndromes, 21 genes involved in mimicking syndromes, and 6 potentially new genes. In total, six, five, four and one probands with CNVs in *SPG7, SPG4*, *SPG11*, and *KIF1A* were identified respectively. Several novel genetic and clinical aspects of HSP subtypes were described. For instance, three patients with homozygous *ATP13A2* mutations presented some novel clinical features and one of the mutations, c.2473_2474insAAdelC;p.[Leu825Asnfs*32]), was already reported in a patient with parkinsonism features. Two patients with compound heterozygous variants in a new candidate gene, *SPTAN1*, were identified; one of these variants, c.2572G>T;p.(Ala858Ser), was carried by both unrelated patients. The frequency of heterozygous pathogenic *SPG7* variants (4.8%) among HSP patients was higher than among controls (1.7%; OR 2.88, 95% CI 1.24–6.66, *P* = 0.009). We identified four heterozygous *SPG7* carriers with an additional variant in known HSP genes, compared to zero in controls (OR 19.58, 95% CI 1.05–365.13, *P* = 0.0031), indicating potential digenic inheritance. We further identified four families with heterozygous *SPG7* variants and variants in *SPG7*-interacting genes. Of these, there was especially compelling evidence for epistasis between *SPG7* and *AFG3L2*.

Conclusion: We described the genetic landscape of HSP, as a heterogeneous group of diseases, in Canada and reported some novel genetic and clinical features of the common and very rare HSP subtypes. Our results also challenge the concept of classical monogenic inheritance in HSP, and the current approach can be applied to other Mendelian disease groups.

Key words: Hereditary spastic paraplegia, HSP, genetic diagnosis, neurogenetic

Résumé

Contexte: Les paraplégies spastiques héréditaires (HSP) sont un groupe de maladies neurodégénératives mendéliennes monogéniques rares caractérisées par une spasticité des membres inférieurs avec/sans symptômes supplémentaires. Jusqu'à présent, 86 gènes ou loci responsables ont été décrits pour HSP avec l'émergence du séquençage de nouvelle génération. Cependant, plus de la moitié des patients HSP restent génétiquement non résolus même avec l'utilisation du séquençage de l'exome (ES). Il existe certaines explications pour expliquer une grande proportion de patients génétiquement non diagnostiqués. Cela inclut la limitation de l'ES pour détecter certaines variations, les occurrences de mutations dans les gènes nouveaux ou non-HSP et le concept actuellement trop simplifié de l'héritage génétique des HSP.

Objectifs: L'objectif général de cette étude était de mieux comprendre la HSP et d'améliorer son rendement diagnostique génétique. **1)** Identifier les variantes pathogènes des gènes impliqués dans la HSP au Canada. **2)** Identifier les variantes pathogènes dans les gènes potentiellement nouveaux et non-HSP. **3)** Étudier les preuves de l'hérédité complexe dans les sous-types courants de HSP.

Méthodes: Au total, 608 personnes provenant de 375 familles avec HSP ont été recrutées à travers le Canada. Le séquençage du panel de gènes HSP a été réalisé sur 435 individus. Collectivement, 422 personnes de 294 familles ont été soumis à l'ES. Les données ES de 580 individus ont également été utilisées pour l'analyse en tant que groupe témoin. Parmi les cas non résolus, 52 patients ont subi un séquençage du génome (GS). Les variants suspects ont été validés par séquençage Sanger. Plusieurs outils in silico ont été appliqués pour détecter les variantes pathogènes. Pour la détection

des CNV dans les données ES, ExomeDepth a été appliqué et l'amplification de sonde multiplex dépendante de la ligature a été utilisée pour la validation.

Résultats: Nous avons identifié 256 des 375 cas index avec des variants potentiellement pathogènes sur 33 gènes HSP connus, 19 gènes impliqués dans des syndromes qui se chevauchent, 21 gènes impliqués dans des syndromes imitateurs et 6 gènes potentiellement nouveaux. Au total, six, cinq, quatre et un proposants avec des CNV dans *SPG7*, *SPG4*, *SPG11* et *KIF1A* ont été identifiés. Plusieurs nouveaux aspects génétiques et cliniques des sous-types de HSP ont été décrits. Par exemple, trois patients présentant des mutations homozygotes de l'*ATP13A2* ont présenté de nouvelles caractéristiques cliniques et l'une des mutations, c.2473_2474insAAdelC;p.[Leu825Asnfs*32]), a déjà été signalée chez un patient présentant des caractéristiques parkinsoniennes. Deux patients avec des variantes hétérozygotes composées dans un nouveau gène candidat, *SPTAN1*, ont été identifiés; l'une de ces variantes, c.2572G>T;p.(Ala858Ser), était portée par les deux patients non apparentés. La fréquence des variants SPG7 pathogènes hétérozygotes (4,8%) chez les patients HSP était plus élevée que chez les témoins (1,7%; OR 2,88, IC à 95% 1,24–6,66, P = 0,009). Nous avons identifié quatre porteurs *SPG7* hétérozygotes avec une variante supplémentaire dans les gènes HSP connus, par rapport à zéro chez les témoins (OR 19,58, IC à 95% 1,05–365,13, P = 0,0031), indiquant un héritage digénique potentiel. Nous avons en outre identifié quatre familles avec des variants *SPG7* hétérozygotes et des variants dans les gènes interagissant avec SPG7. Parmi ceux-ci, il y avait des preuves particulièrement convaincantes d'épistasie entre SPG7 et AFG3L2.

Conclusion: Nous avons décrit le paysage génétique de la HSP, en tant que groupe hétérogène de maladies, au Canada et signalé certaines nouvelles caractéristiques génétiques et cliniques des sous-types de HSP courants et très rares. Nos résultats remettent également en question le concept d'hérédité monogénique classique dans les HSP, et l'approche actuelle peut être appliquée à d'autres groupes de maladies mendéliennes.

Mots clés: Paraplégie spastique héréditaire, HSP, diagnostic génétique, neurogénétique

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Contribution to Original Knowledge

The work presented in this thesis represents an original contribution to the genetics of hereditary spastic paraplegia. The results that are presented in chapter 2 includes the comprehensive genetic findings of all the patients in the cohort. A large proportion of results in this chapter are novel genetic and clinical findings from one of the world's largest HSP cohorts. The results in chapter 3 expand molecular spectrum of a rare type of hereditary spastic paraplegias and provides some novel clinical characteristics. Chapter 4 introduces a new gene in hereditary spastic paraplegia which could be added to the list of genes associated with the disease. Chapter 5 provides evidence for complex inheritance in a type of hereditary spastic paraplegias as a novel insight into the genetics of hereditary spastic paraplegia.

Contribution of Authors

Mehrdad Asghari Estiar wrote and prepared the thesis, and Ziv Gan-Or and Dr. Guy A. Rouleau were involved in constructive feedback, thesis review and critique.

- **Chapter 2. Manuscript 1 (non-published)**

Mehrdad A. Estiar contributed to the original concept of the project, reviewed the patients' files and consulted the CanHSP database for signs, family histories and pedigrees, imaging findings, and genetic analysis and diagnosis. Was involved in genetic data and statistical analyses. Interpreted the results. Performed literature review. Consulted publicly available databases. Performed laboratory experiments such as polymerase chain reaction (PCR), gel electrophoresis, and sample preparation. Wrote the manuscript and reviewed it as it progressed. Eric Yu, Parizad Vargahei, and Etienne Leveille were involved in data analysis, organization, and execution, as well as in manuscript review and critique. Farnaz Asayesh and Jean-Francois Trempe contributed through data analysis and sample preparation. Setareh Ashtiani, Mark Tarnopolsky, Oksana Suchowersky, Nicolas Dupre, Kym M. Boycott, and Grace Yoon, were involved in data collection and patient recruitment. Guy A Rouleau led the project and was involved in data and sample collection and the CanHSP establishment; conception, organization, and overseeing the progression of the research project, as well as manuscript review and critique. Ziv Gan-Or contributed to all stages of the research, from data and sample collection and establishment of the CanHSP database to the conception, design, and organization of the research projects and data analysis, to manuscript review and critique.

- **Chapter 3. Manuscript 2 (published): "Clinical and genetic analysis of ATP13A2 in hereditary spastic paraplegia expands the phenotype." *Molecular Genetics & Genomic Medicine* 8.3 (2020): e1052.**

Mehrdad A Estiar, Guy A Rouleau, and Ziv Gan-Or conceived the study design, Mehrdad A Estiar, Etienne Leveille, Dan Spiegelman, Nicolas Dupre, and Jean-Francois Trempe performed data analysis. Mehrdad A Estiar and Ziv Gan-Or wrote the paper. All authors have read, edited, and approved the final version of the manuscript.

- **Chapter 4. Manuscript 3 (published): "SPTAN1 variants as a potential cause for autosomal recessive hereditary spastic paraplegia." *Journal of Human Genetics* 64.11 (2019): 1145-1151.**

Etienne Leveille and Mehrdad A. Estiar were involved in conception of the research and data analysis. Carried out literature review and interpreted the results. Mehrdad A Estiar and Ziv Gan-Or wrote the manuscript and reviewed it as it progressed. Lynne Krohn, Dan Spiegelman, Alexandre Dionne-Laporte, Simon Veyron, and Jean François Trempe contributed through data analysis. Grace Yoon and Nicolas Dupre were involved in data collection and patient recruitment. Nicolas Dupre was involved in sample collection and patients recruitment. Guy A Rouleau led the project and was involved in patient recruitment, as well as in conception, organization, and manuscript review and critique. Ziv Gan-Or was involved in conception, design, and organization of the research projects, and overseeing the progression of the research project as well as manuscript preparation, review and critique.

- **Chapter 5. Manuscript 4 (published): "Evidence for non-Mendelian inheritance in spastic paraplegia 7." *Movement Disorders* 36.7 (2021): 1664-1675.**

Ziv Gan-Or, Guy A Rouleau, and Mehrdad A Estiar conceived the study design. Ikhlass Haj Salem, Alain Dagher, Grace Yoon, Mark Tornopolsky, Kym Boycott, Nicolas Dupre, Oksana Suchowersky, and Guy A Rouleau were involved in acquisition of data; Mehrdad A Estiar, Ziv Gan-Or, Eric Yu, Etienne Leveille, Jennifer A Ruskey, Kheireddin Mufti, Fulya Akçimen, Etienne Leveille, Dan Spiegelman, Jennifer A Ruskey, Fulya Akçimen, Patrick A Dion, and Jean-Francois Trempe analyzed the data and interpreted the results; Mehrdad A Estiar and Ziv Gan-Or wrote the first draft; All authors have read, edited, and approved the final version of the manuscript. Etienne Leveille, Fulya Akçimen, Jennifer A Ruskey, Patrick A Dion, and Jean-Francois Trempe were involved in administrative, technical, or material support; Guy A. Rouleau and Ziv Gan-Or supervised the study.

List of Figures

List of Tables

List of Abbreviations

AAO	Age at onset
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal dominant
AF	Allele frequency
ALS	Amyotrophic lateral sclerosis
ANNOVAR	Annotate Variation
AR	Autosomal recessive
ATPase	Adenosine triphosphatase
CADD	Combined Annotation Dependent Depletion
CMT	Charcot–Marie–Tooth
CNS	Central Nervous System
CNV	Copy number variation
CP	Cerebral palsy
Cryo-EM	Cryo-electron microscopy
DRD	Dopa-responsive dystonia
EEG	Electroencephalography
EIEE5	Early infantile epileptic encephalopathy-5
EM	Electron microscopy
EMG	Electromyography
ER	Endoplasmic reticulum
ES	Exome sequencing
ExAC	The Exome Aggregation Consortium
GATK	Genome Analysis ToolKit

GERP	Genomic Evolutionary Rate Profiling
GGI	Gene-gene interaction
gnomAD	The Genome Aggregation Database
GO	Gene Ontology
GS	Genome sequencing
GTEx	The genotype-tissue expression
HCA	Hereditary cerebellar ataxias
HSP	Hereditary spastic paraplegia
KRS	Kufor–Rakeb syndrome
LoF	Loss-of-function
LRR	Leucine-rich repeat
MLPA	Multiplex-ligation dependent probe amplification
MRI	Magnetic Resonance Imaging
MTHFR	Methylene tetrahydrofolate reductase
NCL	Neuronal Ceroid Lipofuscinosis
NGS	Next generation sequencing
NHLBI-ESP	The National Heart, Lung, and Blood Institute Exome Sequencing Project
NMD	Nonsense-mediated decay
OMIM	Online Mendelian Inheritance in Man
PCA	Principal component analysis
PD	Parkinson's disease
PNS	Peripheral nervous system
PolyPhen-2	Polymorphism Phenotyping v2
PPI	Protein-protein interaction

RVIS	Residual Variation Intolerance Score
SIFT	Sorting Intolerant From Tolerant
SPG	Spastic paraplegia gene
SPRS	Spastic Paraplegia Rating Scale
TM	Transmembrane
WES	Whole exome sequencing
WGS	Whole genome sequencing

CHAPTER 1: GENERAL INTRODUCTION

BACKGROUND AND LITERATURE REVIEW

Definition and clinical characteristics of hereditary spastic paraplegias (HSPs)

Hereditary spastic paraplegias (HSPs) are a group of rare monogenic Mendelian neurodegenerative disorders affecting the upper motor neurons. The first case series of HSPs was described by Adolf von Strümpell in 1880. Eight years later, Maurice Lorrain published a more detailed report on the clinical study of HSPs. Thus, HSP is also known as Strümpell-Lorrain disease (Engmann *et al.*, 2012). The diagnosis of HSPs is mainly based on the clinical picture. HSP patients present with slowly progressive spasticity and weakness, predominantly in the lower limbs, and often require the use of canes, walkers, or wheelchairs. The current classification of HSP which was proposed about 40 years ago, divides it into two main subtypes, pure (uncomplicated) and complex (complicated). The core clinical features of HSP include lower (and rarely upper) extremity spasticity, weakness, and hyperreflexia, as well as hypertonic urinary bladder disturbance, and mild diminution of lower extremity vibration sensation. Patients with symptoms limited to the core features are diagnosed as pure-HSP. Complex HSP is defined by the presence of additional neurological signs (e.g., ataxia, seizure, dementia, peripheral neuropathy, extrapyramidal disturbance) and/or non-neurological symptoms (e.g., ophthalmological and orthopedic abnormalities, dysmorphic features) on top of the core phenotypic features (de Souza *et al.*, 2017).

Patients who have an early disease age at onset (<35 years) mostly show a slower disease progression and rarely become wheelchair-bound. However, later disease onset is generally associated with the loss of independent ambulation in life's seventh to eighth decade (Harding, 1981; Schady and Sheard, 1990; Polo *et al.*, 1993; McDermott *et al.*, 2000; Shribman *et al.*, 2019). The HSP symptoms may begin at any age from early infancy to late decades of life. The classification of HSPs based on the age at onset has also been described. A proposed diagnostic algorithm exists based upon age at onset, symptoms, and additional information of patients (Shribman *et al.*, 2019) but none of the proposed criteria would perfectly explain the subtypes.

Apart from the clinical classification of HSP, it can be categorized based upon the trait of inheritance. Autosomal dominant (AD), autosomal recessive (AR), and X-linked inheritances have been reported. A large proportion of cases who have no family history are called sporadic. Sporadic cases can result from false paternity, *de novo* cases, reduced penetrance, and uniparental disomy. Pooled prevalence in a systematic review that included 22 studies on HSP and hereditary cerebellar ataxias (HCA), was estimated at around 1:10,000 people. It ranged from 0.5 to 5.5/100,000 and from 0.0 to 5.3/100,000 for AD-HSP and AR-HSP, respectively (Ruano *et al.*, 2014). In Eastern Quebec, the prevalence of HSP ranged from 4.14 to 4.2/100,000, with 2.05/100,000 for AD-HSP and 2.12/100,000 for AR-HSP (Salem *et al.*, 2021).

Pathology of hereditary spastic paraplegias

Few studies have attempted to investigate the neuropathology of HSPs, and the majority of these studies have been conducted in the pre-genetic era (DeLuca *et al.*, 2004;

Blackstone, 2012a). The chronicity and the rarity of the condition may explain the lack of information regarding the neuropathology of HSPs. The core HSP features result from axonal degeneration of the pyramidal motor neurons that control voluntary movements. Extending from layer V of the motor cortex of the cerebrum, pyramidal tract neurons form synaptic connection with the secondary motor neurons which innervate the skeletal muscles. Apart from neurons that are degenerated in HSP, the contribution of glial cells and oligodendrocytes to the pathology of HSP is substantial as some HSP-associated genes are highly expressed in non-neuronal cells (Blackstone, 2012b). Given the cerebellar signs and white matter lesions in most HSP subtypes, it is speculated that HSP's etiopathology is largely unknown, and is not necessarily limited to motor neuron disturbances. Significant white matter lesions in several subtypes raise the possibility that, demyelination or myelin abnormalities could be causal, and not secondary to axonal swelling or degeneration (Fink, 2014). Also, severe spinal cord atrophy has been seen in some HSP subtypes (Pehrson *et al.*, 2018).

The "dying back" of axons could cause neuronal injury in a length-dependent manner in the pyramidal tract, and since longer axons are at greater risk, lower limbs are primarily affected (Behan and Maia, 1974; Kevenaar and Hoogenraad, 2015). As mentioned earlier, in complex HSP, other parts of the nervous system, as well as extra-neurological systems could be involved (Elsayed *et al.*, 2021b). This could arise from the degeneration of the cell bodies of interneurons with shorter axons as well, and the dying-back hypothesis loses its prominence (Elsayed *et al.*, 2021b).

In summary, the currently limited neuropathological descriptions cannot explain all the neurologic manifestations in HSPs such as dementia, neuropathy, and ataxia - as such,

further neuropathological studies are required. However, caution is necessary when reporting the age-dependent observations in neuropathological findings of HSPs as the disease usually does not shorten lifespan and postmortem samples are mainly collected from elderly donors (Fink, 2013).

Underlying molecular mechanisms of hereditary spastic paraplegia

HSPs can also be categorized according to the underlying molecular pathogenetic mechanisms. However, in some HSP subtypes, various pathophysiological mechanisms are involved and determining the exact mechanism is challenging and, in some cases, not feasible. Among the reported cellular processes and pathways, organelle shaping (e.g. morphogenesis of the endoplasmic reticulum), intracellular trafficking (e.g. axonal transport), metabolism (e.g. lipid metabolism), myelination, recycling/degradation, development, and mitochondrial functions are the most commonly reported pathways (Elsayed *et al.*, 2021b). This diversity of pathways in the pathogenesis of HSP make the puzzle of HSPs even more complex. A recent study integrating protein networks and machine learning for disease stratification supports the notion that HSPs should be considered as transportopathies owing to the importance of ‘‘organelle shaping and biogenesis’’ and ‘‘membrane cargo and trafficking,’’ in the etiopathogenesis of HSPs (Vavouraki *et al.*, 2021).

Spastin encoded by the most frequent mutated HSP gene, is an AAA ATPase that catalyzes internal breaks in microtubules. One of the isoforms of spastin interacts with atlastin-1 which is GTPase and is encoded by the second most commonly mutated AD-HSP gene, to sculpt the unique features of the tubular endoplasmic reticulum by

generating polygonal appearance (Blackstone, 2018). The continuous tubular endoplasmic reticulum structure through long axons is essential for axonal transport of newly synthesized lipids and proteins. Spastin and atlastin-1 along with other HSP-associated proteins such as REEP1 and REEP2 play a key role in the function of the endoplasmic reticulum, and their disruptions result in the dysfunction of multiple cellular processes such as axonal transport, endoplasmic reticulum shaping, formation of lipid droplets, and microtubule-related pathways (Blackstone, 2018; Elsayed *et al.*, 2021b). It is thus recently suggested that disturbances of one cellular process or organelle could have a significant effect on other processes (Figure 1).

Paraplegin encoded by the most common AR-HSP gene, is a component of mitochondrial AAA proteases and is mainly engaged in mitochondrial-related pathways. However, mitochondrial morphological abnormalities can also alter regular axonal transport due to traffic jam in the axons (Ferreirinha *et al.*, 2004). Mitochondrial impairment is seen in axons' distal regions prior to axonal degeneration. Therefore, axonal degeneration may be secondary to mitochondrial dysfunction which results in the accumulation of neurofilaments and organelles (Figure 1) (Ferreirinha *et al.*, 2004), similar to spatacsin encoded by the second most common AR-HSP gene (Güner *et al.*, 2021).

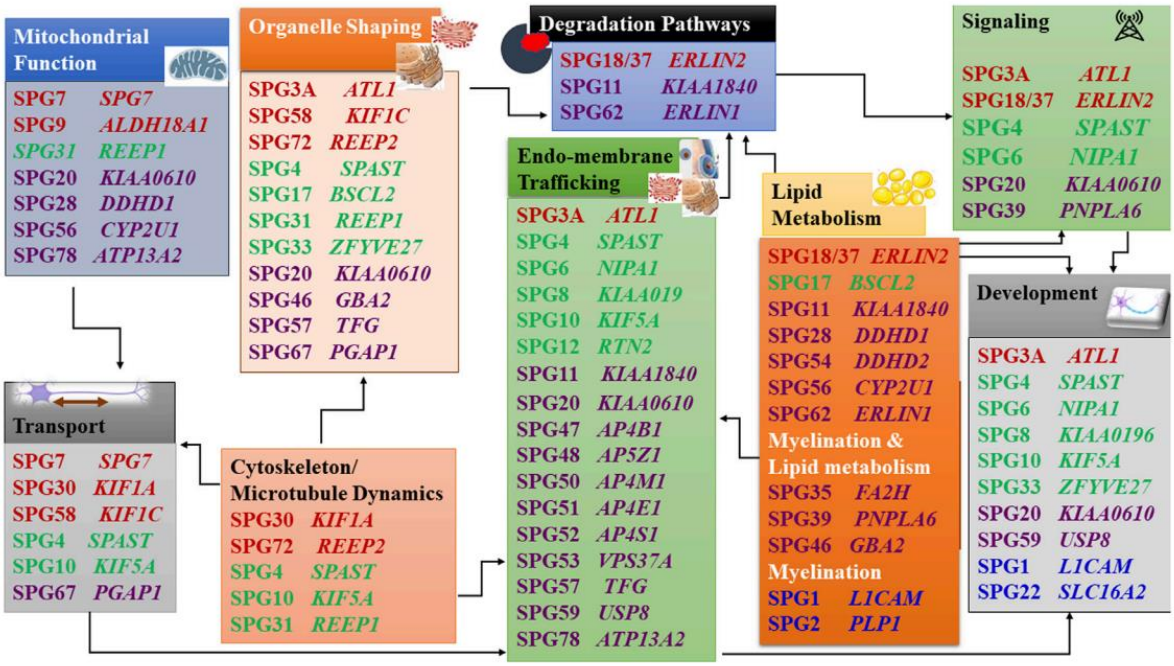

Figure 1. Intersections of HSP pathogenetic mechanisms highlighting the interplay between pathways. Font color codes correspond to various patterns of inheritance: AD HSP (green), AR HSP (violet), X-linked recessive (Blue), and Mixed AR/AD inheritance (red). Reproduced from (Elsayed *et al.*, 2021a). This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY).

Genotype-phenotype correlation analysis recently suggested that AR-HSP proteins mainly engage in degradation, endomembrane trafficking, myelination, and metabolic pathways while microtubule dynamics, development, active cellular transport, and organelle morphology and shaping are highly enriched in AD-HSP related proteins (Elsayed *et al.*, 2021b).

Impaired autophagic lysosomal reformation process leads to excessive lysosomal enlargement and autophagosome accumulation that is often seen in the lack of spatacsin which is encoded by the highest frequently mutated AR-HSP gene. Defects in the autophagy-lysosomal machinery are cumulative and progressive and probably due to secretory vesicle formation failure (Pozner *et al.*, 2020).

Therapeutic options of hereditary spastic paraplegias

Thus far, there are no specific treatments that could reverse or stop neuron degeneration. All the available treatments are symptomatic and are mainly prescribed to improve muscle strength, agility and balance, and also reduce spasticity. Physiotherapy, ankle-foot orthotics, heel raises and anti-spastic drugs such as botulinum toxin injections, tizanidine, baclofen, dantrolene and solifenacin are examples of current recommendations for HSP patients (Hedera, 2021). Abnormal bladder function is treated with trospium chlorure or oxybutynin chlorhydrate. Exergames and dancing are suggested by clinicians to improve gait and motor capacities.

In contrast to the common trend, treating all HSPs with a single drug is probably not possible; particularly due to the various pathophysiological mechanisms implicated in HSPs and multiple processes involved in the etiopathogenesis of one subtype. Another challenge of drug development could be the lack of biomarkers. The available biomarkers in HSPs are demonstrated in Table 1.

Table 1. Biomarkers identified in HSP entities.

Mutated gene	HSP subtype	Biomarker	References
CYP7B1	SPG5	increased levels of 25- and 27-hydroxycholesterol in plasma and cerebrospinal fluid	(Schöls *et al.*, 2017; Prestsæter *et al.*, 2020)
ALDH18A1	SPG9	Low levels of plasma ornithine, citrulline, arginine and proline	(Coutelier *et al.*, 2015)
GBA2	SPG46	Accumulation of glucosylceramide	(Sultana *et al.*, 2015; Malekkou *et al.*, 2018)
B4GALNT1	SPG26	Absence of gangliosides	(Boukhris *et al.*, 2013; Trinchera *et al.*, 2018)
PCYT2	SPG82	Accumulation of etherlipids	(Vaz *et al.*, 2019)

There is also no gene therapy for HSP. However, a genetic diagnosis may lead to a successful management implication. Physiopathological studies reported some potential therapeutic agents experimented on motor neurons derived from induced pluripotent stem cells and animal models of the most common HSP subtype, HSP subtype 4 (Wali *et al.*, 2018; Lallemant-Dudek *et al.*, 2021). Microtubule-targeting drugs have been suggested to be promising for the treatment of SPG4, caused by *SPAST* variants. For instance, vinblastine has been shown to result in disease phenotype amelioration in the SPG4 Drosophila model (Orso *et al.*, 2005). Microtubule-targeting drugs have also been

suggested to reverse axonal swelling seen in the cortical neurons of the SPG4 mouse model (Fassier *et al.*, 2013). In the human olfactory neurosphere-derived cells derived from patients, *SPAST* variants cause decreased peroxisome trafficking speed, as well as reduced levels of acetylated α-tubulin which is a marker of stabilized microtubules. vinblastine, taxol, noscapine, and epothilone D, which are tubulin-binding drugs, may cause increased acetylated alpha tubulin, and, as a result, normalize axonal transport which is the main mechanism involved in SPG4 (Fan *et al.*, 2014a; Wali *et al.*, 2018; Saputra and Kumar, 2021).

Among HSP subtypes that directly affect autophagy and lysosomal pathways, Miglustat has successfully been used in animal models to decrease the accumulation of gangliosides (Boutry *et al.*, 2018). In clinical trials, cholesterol-lowering medications such as statins have been used in HSP subtypes associated with the metabolism of cholesterol, but did not significantly reduce the toxic 27-OH cholesterol levels in the cerebrospinal fluid compared to the plasma (Schöls *et al.*, 2017).

Genes implicated in hereditary spastic paraplegia

The genetic heterogeneity of HSP is matched with clinical and pathophysiological heterogeneity. Since the 1980s, with the advancements in molecular genetic techniques, a few genetic subtypes of HSPs have been identified and numbered sequentially in the order of gene discovery. The acronym SPG means “Spastic Paraplegia Genes” and is assigned to each subtype (i.e. SPG1, SPG2) (Fink, 2014). Variants in each gene/locus cause a distinct subtype. The emergence of next-generation sequencing techniques initiated huge gene discoveries in rare Mendelian disorders including HSP. So far, 86

genes or loci have been described for HSPs (Table 2) according to Online Mendelian Inheritance in Man (OMIM) database. This number includes all known HSP genes/loci that are inherited with AD, AR, and X-linked modes. Recently, mixed traits of inheritance were shown in a handful of HSP subtypes. For instance, SPG9, SPG30, and SPG72 can be caused by both heterozygous and biallelic variants in *ALDH18A1*, *KIF1A,* and *REEP2* respectively (OMIM).

Table 2. Genes and inheritance mode involved in HSPs.

HSPs	Inheritance	Gene name	HSPs	Inheritance	Gene name
SPG01	XL	L1CAM	**SPG44**	AR	GJC2
SPG02	XL	PLP1	**SPG45**	AR	NT5C2
SPG03A	AD	ATL1	**SPG46**	AR	GBA2
SPG04	AD	SPAST	**SPG47**	AR	AP4B1
SPG05A	AR	CYP7B1	**SPG48**	AR	AP5Z1
SPG06	AD	NIPA1	**SPG49**	AR	TECPR2
SPG07	AR	SPG7	**SPG50**	AR	AP4M1
SPG08	AD	WASHC5	**SPG51**	AR	AP4E1
SPG09	AR/AR	ALDH18A1	**SPG52**	AR	AP4S1
SPG10	AD	KIF5A	**SPG53**	AR	VPS37A
SPG11	AR	SPG11	**SPG54**	AR	DDH2

SPG12	AD	RTN2	**SPG55**	AR	C19ORF65R
SPG13	AD	HSPD1	**SPG56**	AR	CYP2U1
SPG14	AR	Gene Locus	**SPG57**	AR	TFG
SPG15	AR	ZFYVE26	**SPG58**	AR	KIF1C
SPG16	XL	Gene Locus	**SPG59**	AR	USP8
SPG17	AD	BSCL2	**SPG60**	AR	WDR48
SPG18	AR	ERLIN2	**SPG61**	AR	ARL6IP1
SPG19	AD	Gene Locus	**SPG62**	AR	ERLIN1
SPG20	AR	SPART	**SPG63**	AR	AMPD2
SPG21	AR	SPG21	**SPG64**	AR	ENTPD1
SPG22	XL	SLC16A2	**SPG65**	AR	NT5C2
SPG23	AR	DSTYK	**SPG66**	AR	ARSI
SPG24	AR	Gene Locus	**SPG67**	AR	PGAP1
SPG25	AR	Gene Locus	**SPG68**	AR	FLRT1
SPG26	AR	B4GALNT1	**SPG69**	AR	RAB3GAP2
SPG27	AR	Gene Locus	**SPG70**	AR	MARS1
SPG28	AR	DDHD1	**SPG71**	AR	ZFR
SPG29	AD	Gene Locus	**SPG72**	AR/AD	REEP2
SPG30	AR/AD	KIF1A	**SPG73**	AD	CPT1C
SPG31	AD	REEP1	**SPG74**	AR	IBA57
SPG32	AD	Gene Locus	**SPG75**	AR	MAG

SPG33	AD	ZFYVE27	**SPG76**	AR	CAPN1
SPG34	XL	SPG34	**SPG77**	AR	FARS2
SPG35	AR	FA2H	**SPG78**	AR	ATP13A
SPG36	AD	SPG36	**SPG79**	AR	UCHL1
SPG37	AD	SPG37	**SPG80**	AD	UBAP1
SPG38	AD	SPG38	**SPG81**	AR	SELENOI
SPG39	AR	PNPLA6	**SPG82**	AR	PCYT2
SPG40	AD	Gene Locus	**SPG83**	AR	HPDL
SPG41	AD	Gene Locus	**SPG84**	AR	PI4KA
SPG42	AD	SLC33A1	**SPG85**	AR	RNF170
SPG43	AR	C10orf12	**SPG86**	AR	ABHD16A

AD-HSP is the most frequent form of HSPs (Skre, 1974; Harding, 1981; Polo *et al.*, 1991; McMonagle *et al.*, 2002; Ruano *et al.*, 2014). Collectively, SPG4, a phenotype that is caused by heterozygous *SPAST* variants, is by far the most frequent subtype of HSPs accounting for 40% of all HSP patients, and 60% of AD cases (Ruano *et al.*, 2014; Boutry *et al.*, 2019; Shribman *et al.*, 2019). Following SPG4, SPG3A (*ATL1*) and SPG31 (*REEP1*) are the next most common AD-HSPs (Boutry *et al.*, 2019; Elsayed *et al.*, 2021b). SPG11 and SPG7 have the highest frequency among AR-HSP subtypes (Elsayed *et al.*, 2021b) followed by SPG5A (*CYP7B1*), and SPG15 (*ZFYVE26)* (Bis-Brewer and Züchner, 2018). There is no detailed information regarding the frequency of HSP subtypes in some regions. It is likely that the frequency differs among various ethnicities.

Differential diagnosis

HSPs are considered phenotypically, pathophysiologically, and genetically an extremely heterogeneous group of disorders. Therefore, a high occurrence of misinterpretation and misdiagnosis can be expected, and molecular testing is the gold standard. There are no curative treatments for HSPs, however, some therapies are available for several overlapping or mimicking disorders. Disorders with pyramidal syndrome as part of their manifestation account for the main diagnostic challenges. Conditions with spastic paraplegia can even be infectious or metabolic in nature. Overlapping disorders impose even more complex diagnostic challenges compared to mimicking disorders, owing to the extensive genetic overlap that HSPs have with the former ones. Some known HSP genes such as *PNPLA6*, *KIAA1840*, *ERLIN1*, *ERLIN2*, and *HSPD1* have been implicated in spastic ataxia, ALS, PLS, and neuropathies (Al-Saif *et al.*, 2012; Synofzik *et al.*, 2014; Kusk *et al.*, 2016; Montecchiani *et al.*, 2016; Tunca *et al.*, 2018a). On the other hand, genes such as *ALS2* and *BICD2* that are known to be involved in other conditions were also reported in HSP patients (Tesson *et al.*, 2015).

Early-onset HSPs could mimic cerebral palsy, the most frequent motor disorder in children with spastic diplegia. In a cohort of patients from Alberta which is part of our Canadian cohort, fourteen HSP patients were initially diagnosed with cerebral palsy (Suchowersky *et al.*, 2021).

Similar to HSPs, symptoms of leukodystrophies can begin at any age and white matter abnormalities can be mild or even absent. On the other hand, more than 20 subtypes of HSP manifest white matter lesions. Therefore, variants in genes associated with

leukodystrophies are expected to be found in HSP cohort studies (Zhang *et al.*, 2021b). Identification of these patients is extremely important due to the current improvement in the prognosis of patients with leukodystrophy using bone marrow transplantation or enzyme replacement therapy. The latter option is currently a promising and relatively effective therapeutic option in metabolic disorders as well. We recently identified three HSP patients from two families with *GCH1* variants which are known to be involved in dopa-responsive dystonia (Varghaei *et al.*, 2021). *GCH1* encodes the enzyme GTP cyclohydrolase 1 which plays an essential role in the synthesis of dopamine. There was a significant improvement in the motor functions of our patients following levodopa treatment. Two cerebrotendinous xanthomatosis patients have been reported who were initially diagnosed with HSP (Nicholls *et al.*, 2015; Saute *et al.*, 2015). A high level of cholestanol is observed in these patients and early treatment can reduce cholestanol in the serum and significantly improve the symptoms. Glucose transporter type 1 deficiency syndrome is known to be caused by *SLC2A1* variants and the phenotype results from glucose deficiency in the brain. Three different studies reported complex HSP cases with a final diagnosis of Glucose transporter type 1 deficiency syndrome (Diomedi *et al.*, 2016a; Nicita *et al.*, 2018; Verrotti *et al.*, 2019). Methylene tetrahydrofolate reductase (MTHFR) deficiency is the most frequent genetic cause of hyperhomocysteinemia which causes a high level of homocysteine in the plasma. Three unrelated probands with *MTHFR* variants were found who were successfully treated with betaine (Lossos *et al.*, 2014; Perna *et al.*, 2018).

The genotype and phenotype overlap that exists between metabolic and neurological disorders could ease the possibility of discovering metabolites or biomolecules to follow

up the progression of disease and assess response to treatment in neurological disorders. A few known HSP genes including *ALDH18A1* (SPG9), *CYP7B1* (SPG5), and *DDHD2* (SPG54) encode proteins implicated in various metabolic pathways. Some studies have been conducted to evaluate the accumulation of metabolites in the brain of HSP cases, and subsequently use it as a biomarker.

RATIONALE, HYPOTHESIS, AND OBJECTIVES

Rationale

Identifying the genetic causes of HSPs could be helpful in several ways. It may lead to accurate genetic counseling and as a result, the number of affected members in the next generation will decrease. Subsequently, the socioeconomic burden of the disease can be reduced. From the patients' point of view, the long diagnostic wandering seen in rare disorders could be avoided. Underpinning the genetic determinants will increase our understanding of HSPs and the affected molecular pathways. This may ultimately pave the way for therapeutic options. Accurate genetic diagnosis is essential for ruling out disorders with available treatment, and, in the future, for the potential genotype-targeted therapy. As the number of patients in each subtype increases, hopes for therapeutic options will arise.

Overall, large-scale collaborative efforts are necessary to better address the gap in the knowledge of the genetics of HSPs as a group of rare heterogeneous disorders and to expand the molecular spectrum of the disease. Since 2014, there have been several HSP studies from Europe and Asia resolving the genetic causes of 23-52% of cases (Orsucci *et al.*, 2014; Balicza *et al.*, 2016a; Kara *et al.*, 2016; Lynch *et al.*, 2016b; Schüle *et al.*,

2016b; Morais *et al.*, 2017a; Dong *et al.*, 2018a; Akçakaya *et al.*, 2020; Cui *et al.*, 2020). These studies mostly had a low sample size and focused on the identification of variants within known HSP genes at the time of publication (Table 3).

Table 3. HSP cohort studies.

Study	Detection technique	Country	Diagnosis rate	Year
(Cui *et al.*, 2020)	Targeted seq	China	28.3% 15/53	2020
(Akçakaya *et al.*, 2020)	Targeted seq/ES	Turkey	23.5% 4/17	2020
(Dong *et al.*, 2018b)	Targeted seq/MLPA	China	47.4% 47/99	2018
(Morais *et al.*, 2017a)	Targeted seq/MLPA	Portugal	41.9% 81/193	2017
(Kara *et al.*, 2016)	ES/Sanger seq/MLPA	UK	49.4% 48/97	2016
(Balicza *et al.*, 2016b)	Sanger seq/WES/targeted seq	Hungary	34.4% 20/58	2016
(Schüle *et al.*, 2016b)	Targeted seq/ ES	German	46.2% 240/519	2016
(Lynch *et al.*, 2016a)	Targeted seq	Greece	52.5% 21/40	2016
(Orsucci *et al.*, 2014)	MLPA/Targeted seq	Italy	46.6% 21/45	2014

Some case studies have shown the implication of non-HSP genes in HSP phenotype and involvement of known HSP genes in other conditions (Eymard-Pierre *et al.*, 2002a; Devon *et al.*, 2003; Fan *et al.*, 2014b; Elsayed *et al.*, 2019a). Thus, it is important to screen all the genes related to the phenotype. Furthermore, the role of oligogenic inheritance and modifier alleles in Mendelian disorders has been barely studied. The results from these few studies have changed the classical belief that rare Mendelian disorders follow a

monogenic pattern of inheritance and have suggested complex model instead. This new insight has resulted in achieving a high rate of genetic diagnoses in rare Mendelian disorders (Posey, 2019) and may lead to identification of medically actionable variants. Complex inheritance could complicate counseling and explain some failures of clinical trials. Moreover, known HSP genes encode proteins that are implicated in various biological pathways and that interplay between multiple cellular processes. The heterogeneous nature of HSP provides an opportunity to discover new genes by applying protein-protein network and pathway enrichment analysis approaches. Similar approaches have been widely applied to study complex neurodegenerative disorders (Manzoni *et al.*, 2020). A part of the gap that exists in the genetic diagnosis of, and in the studies on HSP can be explained by the limitations of applied techniques and tools. A variety of techniques and tools are required to identify and/or confirm the disease-causing alterations that are missed by a single tool or method. Although exome sequencing (ES) has solved some of the limitations of targeted or panel sequencing by providing the possibility of screening all relevant genes, discovering new genes, and identifying oligogenism, it has its own limitations. One concern is the detection of large structural variants. Numerous deletions within different HSP genes, such as *SPAST*, *ATL1*, *NIPA1*, *REEP1*, *DSTYK*, and *SPG7*, have been described (Varga *et al.*, 2012; Lee *et al.*, 2017). Detecting copy number variations (CNVs) as a part of large structural variants, from ES is always challenging and unreliable. Thus, a secondary method as a gold standard is often required to confirm these alterations. Another concern is the limitation of ES in detecting deep intronic variants. Recent studies provided evidence for the presence of secondary alleles within deep non-coding regions of *POLR3A* in HSP and cerebellar

ataxia patients (Minnerop *et al.*, 2017b; Minnerop *et al.*, 2019). Similarly, a recent case report revealed compound heterozygous missense and deep intronic variants in *SPG7* (Verdura et al., 2020).

Therefore, we aimed to conduct a study to decipher the genetics of HSPs in Canada by expanding the current molecular findings, and to provide novel insights by overcoming the limitations of current HSP cohort studies.

Hypothesis

The genetic etiology of HSPs is heterogeneous and to uncover it, multiple genetic aspects of the disease including the role of all types of genetic alterations, complex inheritance, non-HSP genes, and new genes should be considered in its analyses.

Objectives

General Objective

To expand the molecular and clinical spectrum of HSPs in Canada and to determine the genetic causes of HSPs and the unknown aspects of the disease.

Specific Objectives

i) To identify disease-causing variations in known HSP genes in Canada

ii) To study evidence for non-Mendelian inheritance in the most common AR-HSP subtype

iii) To identify disease-causing variants in potential new genes implicated in HSP

CHAPTER 2: MANUSCRIPT 1 (non-published)

The genetic landscape of hereditary spastic paraplegia in Canada

Mehrdad A Estiar[1,2], Eric Yu[1,2], Parizad Varghaei[2,3], Etienne Leveille[4], Setareh Ashtiani[5], Lauren Brady[6], Farnaz Asayesh[2], Nicolas Dupré[7], Kym M. Boycott[8], Oksana Suchowersky[9], Grace Yoon[10], Mark Tarnopolsky[6], Jean François Trempe[11], Guy Rouleau[1,2,12*], Ziv Gan-Or[1,2,12*]

1. Department of Human Genetics, McGill University, Montréal, Quebec, Canada
2. Montreal Neurological Institute and Hospital, McGill University, Montréal, Quebec, Canada
3. Division of Experimental Medicine, Department of Medicine, McGill University, Montréal, Quebec, Canada
4. Faculty of Medicine, McGill University, Montréal, Quebec, Canada
5. Alberta Children's Hospital, Medical Genetics, Calgary, Alberta, Canada.
6. Department of Pediatrics and Medicine, McMaster University, Hamilton, Canada d
7. Division of Neurosciences, CHU de Québec, Université Laval, Québec City, QC, Canada
8. Department of Genetics, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada
9. Department of Medicine, Division of Neurology, University of Alberta, Edmonton, Alberta, Canada
10. Division of Neurology, Department of Paediatrics, University of Toronto, The Hospital for Sick Children, Toronto, Canada
11. Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada
12. Department of Neurology and Neurosurgery, McGill University, Montréal, Quebec, Canada

Correspondence to:

Guy A. Rouleau,

Director, The Neuro (Montréal Neurological Institute-hospital),

Address: 3801, University Street, Office 636

Montréal, Québec H3A 2B4

Phone: +1-514-398-2690,

Fax. +1-514 398-8248

Email: guy.rouleau@mcgill.ca

Ziv Gan-Or

Department of Neurology and Neurosurgery

McGill University

1033 Pine Avenue, West,

Ludmer Pavilion, room 312

Montreal, QC, H3A 1A1,

Phone: +1-514-398-5845

Fax. +1-514 398-8248

Email: ziv.gan-or@mcgill.ca

Abstract

Hereditary spastic paraplegias (HSPs) are a group of rare monogenic Mendelian disorders unified by predominant lower limb spasticity with or without additional symptoms. Thus far, more than 80 causative genes/loci have been described for HSP. The HSP cohort studies thus far mainly had a low sample size and genetic diagnosis rate. There are some potential explanations to account for the large proportion of genetically unsolved cases. We aimed to expand the molecular spectrum of HSP by presenting a large Canadian cohort of HSP patients, CanHSP, and to increase the genetic diagnostic yield. A total of 375 HSP families with a total of 608 individuals met the inclusion criteria and were included in the study. In total, 250 probands (435 individuals) went through panel sequencing, resulting in the genetic diagnosis of 81 probands (186 individuals). The remaining 169 probands (249 individuals), as well as additional 125 HSP probands (173 individuals) who did not go through panel sequencing and 580 unrelated control individuals were analyzed by exome sequencing (ES). Following the analysis of ES data and applying multiple *in silico* tools, a further 173 probands were genetically solved, and 52 unsolved patients were selected to perform genome sequencing (GS). A total of 256 index cases (68.2%) were genetically diagnosed, among which 196 harbored variants in known HSP genes, 31 overlapping, 22 mimicking, and 6 novel candidate HSP genes. A total of 16 CNVs were detected across *SPG7*, *SPAST, SPG11* and *KIF1A*. There were 11 patients with potentially pathogenic variants in two distinct relevant genes. Two potentially new genes *AP5B1* and *CAPNS2* were identified. This study exemplifies the complex genetic landscape for HSP by studying one of the world's largest cohorts of the

disorders. Additional studies are required to validate the detected candidate genes in HSP.

Keywords: Hereditary spastic paraplegia, whole exome sequencing, copy number variations, genetic diagnosis, Mendelian disorder

Introduction

Hereditary spastic paraplegias (HSPs) are a heterogeneous group of rare monogenic Mendelian neurodegenerative disorders characterized by predominant symmetrical lower extremity spasticity and weakness (Fink, 2014). A systematic review and meta-analysis of HSP prevalence studies estimated a range from 0.1-9.6/100.000 (Ruano *et al.*, 2014) in different populations. Although distal degeneration of descending corticospinal and sensory tracts is the main pathologic finding of HSP (DeLuca *et al.*, 2004), there is a lack of detailed pathological knowledge so far, probably due to its chronicity and rarity.

While HSP can be classified according to the clinical presentation, genetic types, mode of inheritance, pathophysiological molecular mechanism, and age of onset (de Souza *et al.*, 2017), none of the classifications are appropriate for disease description (Elsayed *et al.*, 2019b). In clinical classification, patients who exclusively manifest the core HSP symptoms including lower limb spasticity, weakness, and hyperreflexia, as well as variable hypertonic urinary disturbances and ankle clonus, are classified as having pure-HSP. The presentation of other neurological and non-neurological signs in addition to the core features to make the diagnosis of complex-HSP (Giudice *et al.*, 2014; Chrestian *et al.*, 2017a; de Souza *et al.*, 2017; Yücel-Yılmaz *et al.*, 2018). Due to the wide clinical spectrum, HSP overlaps with neurological and inherited metabolic disorders, as well as myelopathies.

HSPs are associated with diverse pathophysiological molecular mechanisms, as the HSP-related proteins are involved in endoplasmic reticulum morphology, axonal transport, myelination, membrane trafficking, lipid metabolism, and mitochondrial function (Fink, 2014; Hensiek *et al.*, 2015; de Souza *et al.*, 2017). The emergence of next-

generation sequencing revealed that from the genetic point of view, HSPs are even more complex with more than 80 causative genes and loci. Several subtypes were identified in merely a handful of HSP families and therefore, are not clinically and genetically well-described thus far. Even the common subtypes' frequencies vary depending on geographical region (Lynch *et al.*, 2016b; Dong *et al.*, 2018a).

Due to the heterogeneity, correlating clinical features with its specific subtype has been challenging (Lu *et al.*, 2018). Furthermore, variants in some known HSP genes can also cause non-HSP diseases (Synofzik *et al.*, 2013; Montecchiani *et al.*, 2015; Tunca *et al.*, 2018b) and variants in non-HSP genes were reported in HSP (Synofzik *et al.*, 2016; Simone *et al.*, 2018). Thus, studies from large cohorts at an exome- or genome-wide level are required to investigate the genetic etiology of HSP as a key step toward a better understanding of HSP and overlapping or mimicking syndromes. The previous HSP studies mainly focused on identifying single nucleotide variants in protein-coding regions by screening only known/common HSP genes in cohorts with a relatively low sample of patients (Ishiura *et al.*, 2014; Balicza *et al.*, 2016b; Lynch *et al.*, 2016c; Elert-Dobkowska *et al.*, 2019a). Some proportion of the unsolved cases in these studies can be explained by the fact that only known HSP genes were screened. Other potential explanations could include the limitations of the techniques that were used, the occurrence of variants in new genes, and not accounting for the possibility of a complex inheritance model. In the present study, we conducted a cross-sectional study in one of the world's largest HSP cohorts, CanHSP, to further broaden the mutational spectrum of HSP, and to improve its genetic diagnostic yield.

Materials and methods

Subjects

A total of 608 individuals from 375 families were recruited across Canada including 326 individuals (161 families) from Montreal, 38 (25 families) from Quebec City, 44 (41 families) from Ottawa, 86 (65 families) from Edmonton/Calgary, 79 (54 families) from Toronto, 29 (24 families) from Hamilton, and 6 (5 families) from Vancouver. In total, 435 individuals (250 probands) were panel sequenced and 422 individuals (294 probands) went through ES. Additional ES data of 580 unrelated individuals out of 1,175 individuals were used as an in-house control. These controls included healthy individuals and individuals with non-movement disorders (Supplementary Table 2.1).

Pedigrees suggested autosomal recessive (AR)-HSP in 150, autosomal dominant (AD)-HSP in 128, sporadic in 31, and X-linked-HSP in 13 families. The mode of inheritance in 51 families remained unknown.

The diagnosis was based on previously published diagnostic criteria for HSP (Gasser *et al.*, 2010). Standardized clinical assessments were carried out, and included demographic features, pedigree and family history, developmental history, and symptoms of HSP (Supplementary Table 2.2). For a subset of probands (n=180), disability was evaluated using the Spastic Paraplegia Rating Scale (SPRS). Brain and total spine MRIs were done for 240 and 227 probands respectively.

Genotyping and sequencing

The extraction of DNA was performed according to standard procedures. Exome capture was performed with SureSelect Human All Exon V4, V5, TruSeq Exome, and Nextera Rapid Capture Exomes (Illumina) kits. ES was performed on a HiSeq 2000 or 2500 machine (Illumina). For alignment, variant calling, and annotation, Burrows-Wheeler Aligner, Genome Analysis Toolkit, and ANNOVAR were used respectively. In the next step, data on the detected variants were extracted from The Genome Aggregation Database (gnomAD), The Exome Aggregation Consortium (ExAC) Browser, dbSNP, the National Heart, Lung, and Blood Institute Exome Sequencing Project (NHLBI-ESP), and the 1000 Genomes Project. To predict the pathogenicity and functional effects of the variants and genomic evolutionary rate profiling, ClinVar, VarSome, CADD, RVIS, MutationTaster (Schwarz *et al.*, 2014), SIFT (Kumar *et al.*, 2009), PolyPhen-2 (Adzhubei *et al.*, 2010), PhyloP (Pollard *et al.*, 2010), and GERP++ (Davydov *et al.*, 2010) were applied. The initial inclusion criterion for variants was an allele frequency of less than 0.01. Further filtering was done based on predicted deleterious effects and conservation. Suspected variants were verified by Integrative Genomics Viewer and validated by Sanger sequencing (Applied Biosystem's 3730xl DNA Analyzer technology) after designing specific primers to amplify DNA from all available affected and unaffected members of a family.

Copy number variation analysis

To call CNVs in HSP patients, we used ExomeDepth which is highly suited to detect rare CNV calls for rare Mendelian disorders. We removed controls suspected to carry a CNV in any HSP gene from the reference set. A total of 300 control samples were used by

ExomeDepth subsequently. Each exome data of the test sample was compared to the best set of reference data out of 300 exome data, selected by the tool according to the correlation of the coverage for each probe between the test and reference(s) with an average of 6.6 references per test. Following the selection of the reference set, we ran the CallCNV function in ExomeDepth with default settings. A filter for samples with a correlation above 0.97 is applied to remove false positives. To validate the CNVs, we used the SALSA MLPA P211 HSP region probemix 100 rxn and SALSA MLPA Probemix P213 HSP mix-2 25rxn kits and positive/negative controls according to the manufacturer instructions (MRC Holland), which is the gold standard method for CNV detection, and analyzed by the Coffalyzer software.

Software and tools for analysis

For the prediction of protein domains and functional sites, the pathogenicity of CNVs and sequence alignment, InterPro (Mitchell *et al.*, 2019), X-CNV (Zhang *et al.*, 2021a), and ClustalOmega (Sievers *et al.*, 2011) tools were used respectively. Moreover, we used Somalier (Pedersen *et al.*, 2020) to test relatedness of individuals when a suspected variant was not co-segregated with the disease and prior to running ExomeDepth (Plagnol *et al.*, 2012) to remove relatives in controls. For protein-protein interactions (PPI), gene-gene interactions (GGI), and, Gene Ontology and pathway enrichment analysis, we used STRING (Szklarczyk *et al.*, 2019), GeneMANIA (Warde-Farley *et al.*, 2010) and g:Profiler (Raudvere *et al.*, 2019) respectively. The structure of full-length human CAPN1 (a.a. 1-714) bound to human CAPNS2 (a.a. 1-248) was predicted using the ColabFold platform (Mirdita *et al.*, 2022), based on the AlphaFold2 algorithm (Jumper *et al.*, 2021). Briefly,

the two sequences were submitted as a heterodimer using the MMseqs2 multiple sequence alignment and other default options. Five models were generated, which were highly similar (<0.4 Å rmsd). Likewise, the structure of human AP-5 β (AP5B1, a.a. 392-650) bound to AP-5 ζ (AP5Z1, a.a. 441-807) and AP-5 μ (AP5M1, a.a. 192-490) was predicted using the ColabFold platform. While the μ subunit did not converge, the coordinates for the β and ζ subunits superposed with less than 0.25 Å rmsd for all five models. The atomic coordinates for the AP-2 structure were downloaded from the Protein Data Bank (ID 6owo). The effect induced by each mutation was evaluated using the "mutagenesis" toolbox in The PyMOL Molecular Graphics System, Version 2.4.0 Schrödinger, LLC.

Results

Of the 375 HSP families who were included in the analysis, 250 index cases were sequenced for a panel of known HSP genes. Of those, 81 (32.4%) were genetically diagnosed. The remaining 169 index cases, plus 125 index cases without a prior genetic test, underwent ES (Figure 1).

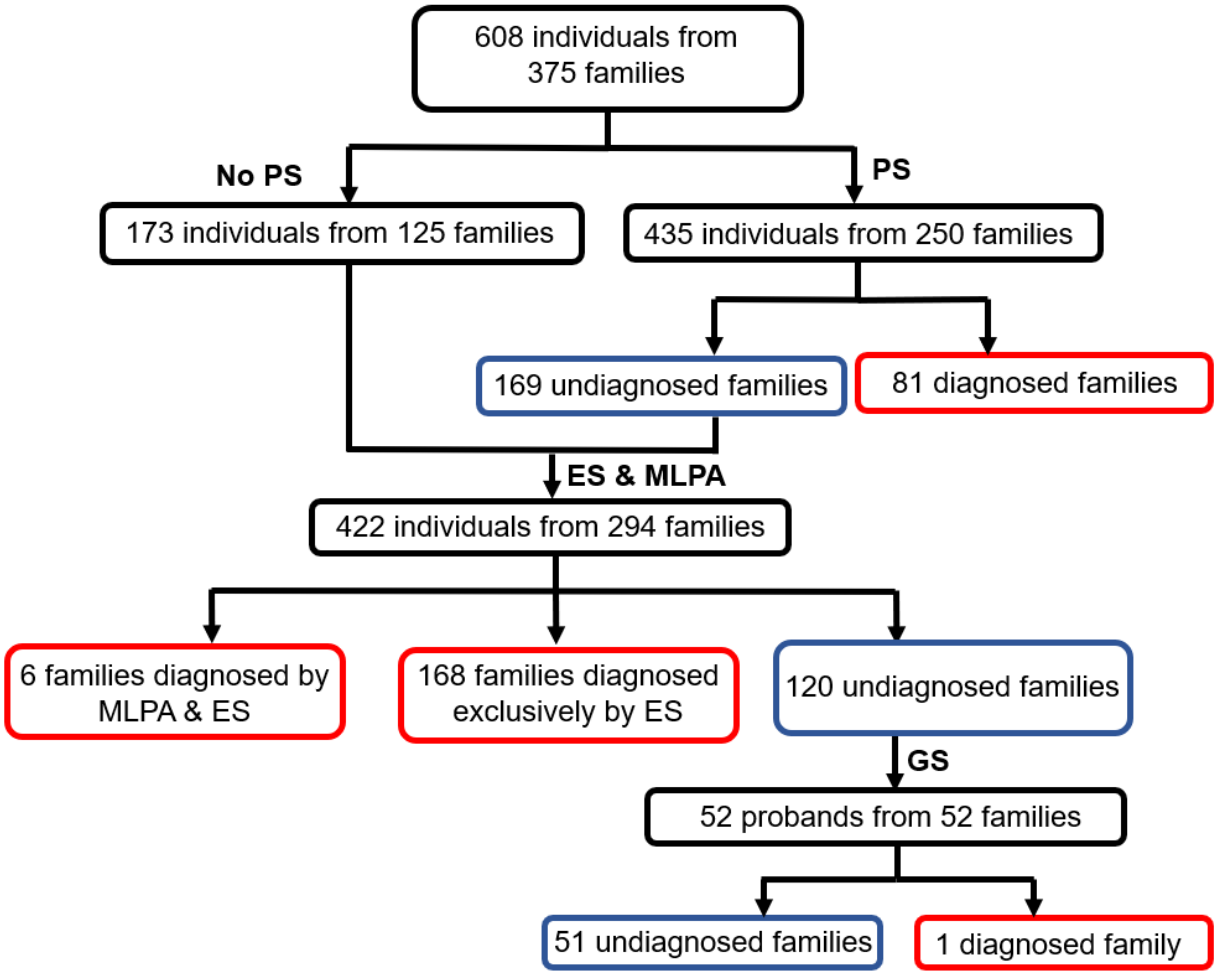

Figure 1. Flow diagram outlining the recruitment of HSP patients and the genetic diagnostic research process. The blue and red rectangles indicate undiagnosed and diagnosed patients respectively. PS: panel sequencing; ES: exome sequencing; MLPA: Multiplex-ligation dependent probe amplification; GS: genome sequencing.

Collectively, of 375 families, 256 index cases (68.2%) were identified with rare and potentially pathogenic variants across 33 known HSP genes, 19 genes implicated in overlapping syndromes, 21 genes involved in mimicking syndromes and 6 potentially new genes.

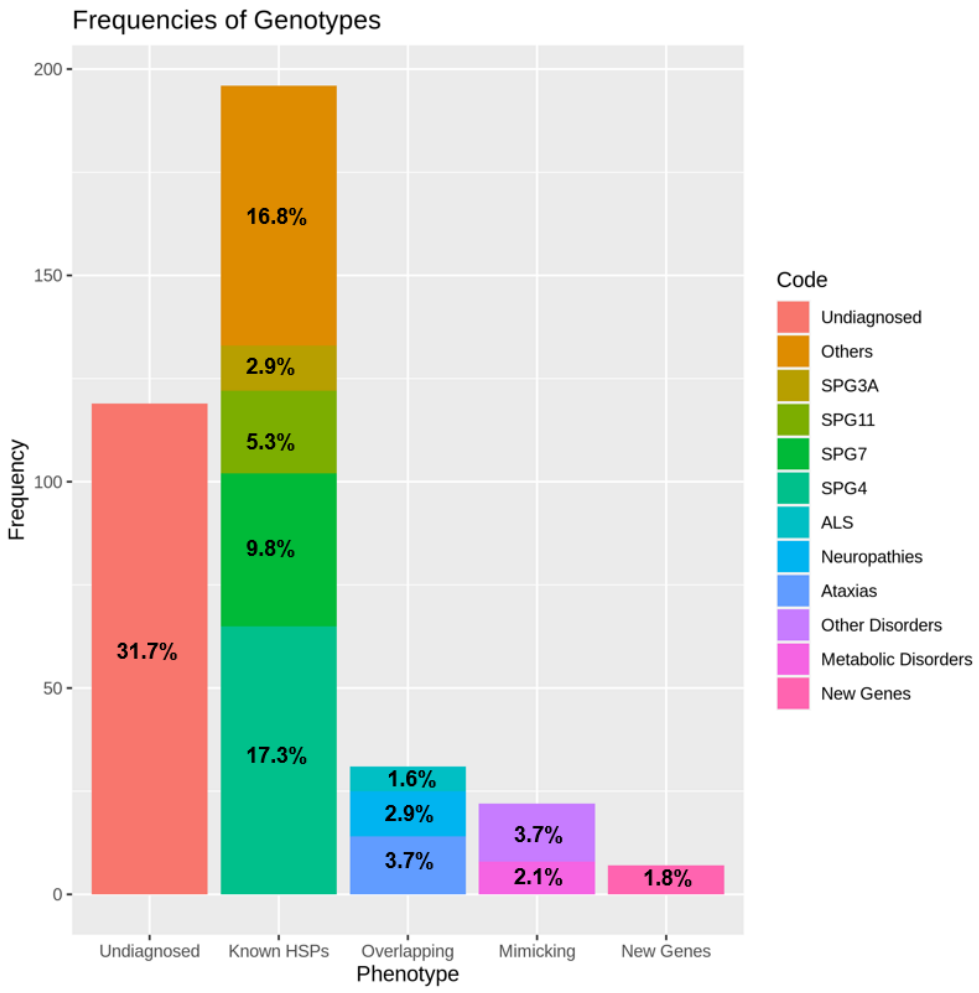

Figure 2. Frequency of genotypes.

Variants in known HSP genes

Among the 196 index cases with variants in known HSP genes, 248 genetic alterations were identified, 119 of which are not reported in gnomAD. Collectively, 138 missense, 31 nonsense, 23 splice site, 32 frameshift indel, 6 in-frame indel, 1 start loss, 2 synonymous variants, and 16 CNVs, were detected (Supplementary Table 2.3). *SPAST* and *SPG7* were the most frequently mutated genes in our cohort and were identified in 27.5% (103/375) of all the studied subjects.

To detect CNVs as a secondary *SPG7* allele, the heterozygous carriers of *SPG7* were analyzed by MLPA. Interestingly, we found three patients who carried pathogenic SNVs, p.(Ala510Val), p.(Leu705LeufsTer100), p.(Lys716Glu), with a deletion of *SPG7* exon 16. We applied ExomeDepth which is the most reliable tool to detect CNVs in ES data in rare Mendelian disorders (Plagnol *et al.*, 2012; Marchuk *et al.*, 2018), and detected 13 patients with potential *SPAST* CNVs. After validation by MLPA, 3 CNVs were confirmed including a large deletion of exons 16 and 17 in one patient, and two patients with deletions of exon 1, as previously described (Varghaei *et al.*, 2022). To further detect CNVs in uncommon HSP subtypes, of 120 genetically undiagnosed cases, 52 samples went through GS. We identified a patient with a loss of exon 12 in *KIF1A* (chr2:240771834-240775833). We also found a rare synonymous *REEP2*:c.540C>T;(p.Ser180=) variant but it was not included in our analysis as there is no evidence regarding its pathogenicity. We could not find any previously reported pathogenic intronic variant.

Genes implicated in overlapping disorders

We sought to determine if the samples that underwent ES (n=422) carry variants in genes associated with diseases that may present with spastic paraplegia/quadriplegia as part of their phenotypic spectrum. In total, we identified 31 index cases with variants across 19 genes (Supplementary Table 2.3). The GO and pathway enrichment analysis of the 19 proteins corresponding to these genes showed that they closely interact with known HSP proteins mainly in axon-related biological pathways such as axonal and axodendritic transport, axonogenesis, and axon development (Supplementary Table 2.4). Of 40 detected variants, 22 are not reported in gnomAD. Of all the detected variants, 29 are missense, 6 frameshift indels, 2 nonsense, and 3 splice site variants. GS revealed 4 CNVs in *DNM1L* and *DCC,* predicted as pathogenic and likely pathogenic (Supplementary Table 2.5) by X-CNV prediction tool (Zhang *et al.*, 2021a) but as CNVs are a large source of normal variation, we did not include them in our analysis.

Genes associated with disorders that mimic HSP phenotype

Variants in genes implicated in a phenotype resembling HSPs were previously described in HSP patients (Bajaj *et al.*, 2002; Diomedi *et al.*, 2016a; Leveille *et al.*, 2018; Ciarlariello *et al.*, 2020; Pauly *et al.*, 2021). We identified 22 index cases with rare and potentially pathogenic variants in genes corresponding to mimicking disorders (Table 1). A total of 20 missense, 4 nonsense, 2 frameshift indels, 2 splice site, 1 in-frame indels, and 2 deep intronic variants were detected, 12 of which are not reported in gnomAD. Two index cases with homozygous deep intronic *POLR3A*:c.1909+22G>A and compound heterozygous *POLR3A*:c.1909+22G>A & c.3205C>T:p.(Arg1069Trp) variants were identified, previously was reported as pathogenic (Minnerop *et al.*, 2017b; Ruggiero *et al.*, 2020).

HSP candidate genes

CAPNS2

To further decipher the genetic causes of HSPs, we pooled ES data of the genetically unsolved cases and only focused on very rare/novel biallelic variants. We also prepared a list of candidate genes that interact with HSP genes or there was previously published functional evidence. The homozygous variants in *CAPNS2* and *AP5B1* genes which are not assigned to any disease were identified.

The *CAPNS2*:p.(Val214Ile) variant was carried by two affected members of a Pakistani family (Supplementary Figure 2.1). The AAO in our two patients was under one year and along with core HSP features, both showed progressive cognitive deficits and dystonia at the age of 17 and 19. There is no study reporting variants in *CAPNS2* in any diseases. In a study, homozygosity mapping with the DNA of HSP patients with dystonia and cognitive dysfunction revealed an identical region encompassing *CAPNS2*, *FA2H* (SPG35), and four other genes. Sequencing of these genes revealed a homozygous variant in *FA2H* (Edvardson *et al.*, 2008).

The protein encoded by the *CAPNS2* gene is a small regulatory subunit of the calpain heterodimeric protease complex (Schad *et al.*, 2002). PPI using STRING revealed that CAPNS2, as a subunit of the calpain family, closely interacts with CAPN1 and SPTAN1, two HSP-associated genes, (Supplementary Figure 2.1) in “deregulated CDK5 triggers multiple neurodegenerative pathway” (HAS-8862803), “degradation of the extracellular matrix” (HAS-1474228) and “extracellular matrix organization” (HAS-1474244) pathways. In humans, there are two small subunits (*CAPNS1* and *CAPNS2*), which can each form complexes with the large catalytic subunits calpain-1 (μ, *CAPN1*) or calpain-2 (m, *CAPN2*)

and modulate their activity as a function of calcium concentration. As opposed to the ubiquitously expressed *CAPNS1*, *CAPNS2* expression is restricted to brain axons and presynaptic areas (Friedrich *et al.*, 2004a). While there is no experimental structure of the CAPNS2 protein, it is highly homologous to CAPNS1 (63% sequence identity), for which there are experimental structures bound to the large catalytic subunit CAPN2 (Strobl *et al.*, 2000). Here, we used the ColabFold platform (Mirdita *et al.*, 2022) to predict the heterodimer complex structure of CAPNS2 bound to CAPN1 (Figure 3A). The structure is predicted with high confidence and is highly similar to the CAPNS1-CAPN2 (pdb 1kfu; rmsd 1.9 Å). The residue Val214 is located in the hydrophobic core of CAPNS2 and its sidechain interacts with two helices near a calcium-binding EF-hand (Figure 3B). The mutation p.V214I would create steric clash with Ile127 or Leu147, causing a disturbance in the structure of the EF-hand motif that could disrupt calcium-binding and/or interaction with the large calpain subunit.

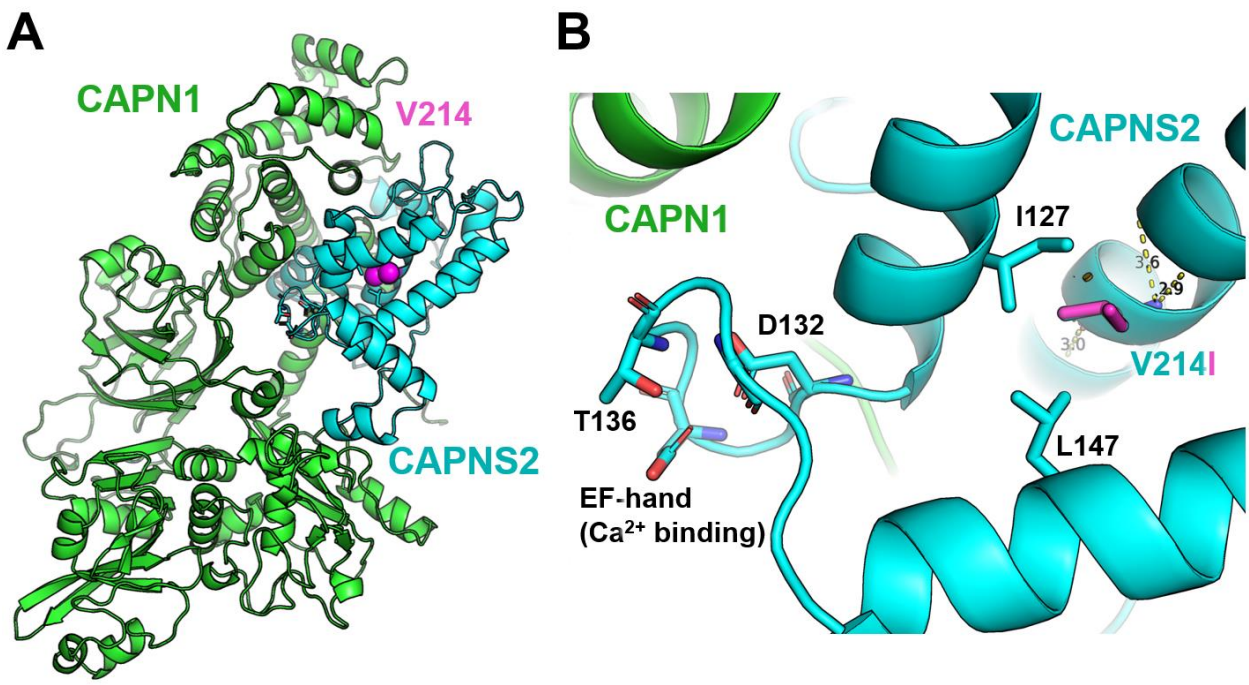

Figure 3. Structural analysis of the *CAPNS2* V214I variant. (A) Predicted structure of the complex formed by the calpain catalytic subunit (CAPN1, green) and the small calpain regulatory subunit 2 (CAPNS2, cyan). The position of Val214 is highlighted in magenta. (B) Zoom-in of the CAPN1:CAPNS2 interface near Val214. The side-chain of Val214 points toward the hydrophobic core of the protein. In silico mutagenesis to an isoleucine results in minor clashes with another hydrophobic core residues, Ile127. This could destabilize the structure of the EF-hand that interacts with CAPN1.

AP5B1

The homozygous *AP5B1*:p.(Arg578Trp) variant was carried by the sole affected member of an Iranian consanguineous family. The index proband started to toe walk at the age of 11. He then started having a progressively slow deterioration in his gait with lower

extremity weakness and spasticity and ankle clonus. The brain and total spine MRI showed cerebellar atrophy and leukodystrophy.

A heterozygous *AP5B1* and *AP5Z1* variants were reported in an HSP patient (Morais *et al.*, 2017b). AP5B1 is the β subunit of the AP-5 adaptor protein complex, which is involved in late endosomal trafficking (Hirst *et al.*, 2011). AP-5 forms a heterotetramer made of two large (ζ, β) a medium (μ), and small (σ) subunits (Hirst *et al.*, 2013b). Knockout of the AP-5 ζ subunit in mammalian cells leads to impaired trafficking of receptors from the endosomes back to the Golgi and accumulation of aberrant endolysosomes, suggesting that the protein is involved in sorting receptors at the late endosomal stage (Hirst *et al.*, 2018b). The AP-5 ζ subunit (*SPG48*) has been previously associated with HSP (Slabicki *et al.*, 2010) (Supplementary Figure 2.1). AP5B1 interacts with ZFYVE26 (SPG15) and SPG11, the depletions of which result in reduced levels of AP5B1 (Chang *et al.*, 2014a). To better understand the impact of the p.(Arg578Trp) missense variant in the AP-5 β subunit, we sought to assess its structure and interactions. There are no experimental structures for the AP-5 subunits. While the AP-5 subunits display low sequence conservation with other adaptor protein complexes (<10%), their tetrameric architecture is conserved, and thus we can learn about their interactions by comparison with other AP structures. The structure of the phosphorylated AP-2 complex bound to adaptor protein NECAP has been determined by cryoelectron microscopy (Partlow *et al.*, 2019) and reveals the global architecture of the complex (Figure 4A). The C-terminus of the β subunit (upstream of the “ear” domain) interacts with the C-terminus of the α subunit (ζ in AP-5) and the C-terminus of the μ subunit. To determine whether AP-5 subunits mediate similar interactions, we used the ColabFold platform (Mirdita *et al.*, 2022) to predict PPI between

the AP-5 β, ζ, and μ subunits. The predicted align error (PAE) plot shows that the interface between the β and ζ subunits is predicted with low error and high confidence, by contrast with interactions with the μ subunit (Figure 4B). In the predicted model, the β and ζ subunits interact via polar and non-polar interactions, with a buried surface area of 5310 Å^2 (Figure 4C). Notably, the sidechain of Arg578 in the β subunit is located at the interface and forms a hydrogen bond with the backbone carbonyl of Arg742 in the ζ subunit. The variant p.(Arg578Trp) would disrupt the hydrogen bond and potentially cause steric clashes (Figure 4D), which would most likely result in the disruption of the interactions between the two subunits.

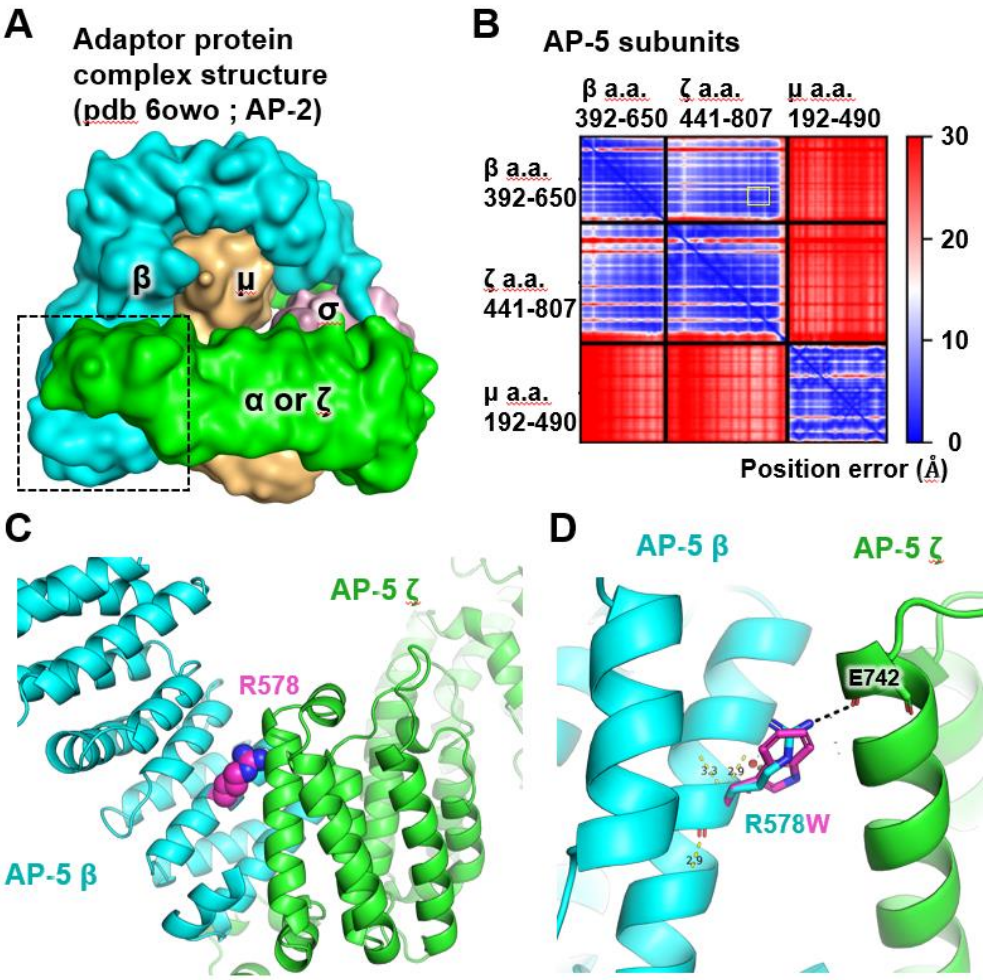

Figure 4. Structural analysis of the *AP5B1* R578W variant. (A) General architecture of the adaptor protein complex. The experimental structure of the AP-2 complex is shown here as an example (pdb 6owo). The β subunit (green) forms a solenoid that interacts with the other subunits of the complex. Notably, the C-terminus of the β subunit interacts with the C-terminus of the the α or ζ subunit (dashed line). (B) ColabFold prediction and positional error of the interactions between the AP-5 β1, ζ1 and μ1 C-termini. The yellow box indicates that the region surrounding Arg578 in the β1 subunit forms a well-defined interface with the ζ1 subunit, but not the μ1 subunit. (C) Cartoon representation of the predicted AP-5 β1: ζ1 complex. The position of Arg578 is highlighted in magenta. (D) Zoom-in of the AP-5 β1: ζ1 interface. In silico mutagenesis of β1 R578W results in minor

clashes and removal of a polar interaction with the backbone carbonyl of Glu742 in the ζ1 subunit.

Patients with mixed or complex inheritances

We identified 11 patients with potentially pathogenic variants in two distinct genes (Table 1). None of the controls carried rare concurrent variants in these genes. We also identified a patient with three pathogenic *SACS* variants, p.(Pro3652Thr) & p.(Glu141AspfsTer42) & p.(Arg3636Gln). In addition, 4 patients belonging in two families with homozygous variants in *ATL1*:p.(Arg217Ter) and *SPAST:*p.(Tyr51Ter) were identified.

Table 1. HSP patients with variants within two distinct genes.

No	ID	Gene 1	Zygosity	Nucleotide change	Protein change	AF	Gene 2	Zygosity	Nucleotide change	Protein change	AF
1	5-026	*NT5C2* (NM_012229.4)	Homo	c.1481A>G	p.His494Arg	4.01e-6	*FA2H* (NM_024306.5)	Homo	c.649G>A	p.Gly217Arg	7.97e-6
2	1-015	*ATL1* (ENST00000358385.6)	Homo	c.649C>T	p.Arg217Ter	3.98e-6	*WASHC5* (NM_014846.4)	Het	c.595C>T	p.Pro199Ser	NR
3	4-011	*REEP1* (NM_022912.3)	Het	c.377dup	p.Leu126PhefsTer61	NR	*KIF5A* (NM_004984.4)	Het	c.2939C>T	p.Ala980Val	7.95e-6
4	4-058	*SPG7* (NM_003119.4)	Homo	c.1529C>T	p.Ala510Val	2.90e-3	*KIF1A* (NM_001244008.2)	Het	c.3616C>T	p.Arg1206Trp	1.08e-5
5	1-165	*SPAST* (NM_014946.4)	Het	c.1378C>T	p.Arg460Cys	NR	*AFG3L2* (NM_006796.3)	Het	c.1255G>A	p.Gly419Ser	NR
6	2-047	*DYNC1H1* (NM_001376.5)	Het	c.9656G>A	p.Arg3219His	3.98e-6	*PDGFB* (NM_002608.4)	Het	c.64-3C>T	-	4.01e-6
7	5-031	*HSPD1* (NM_002156.5)	Het	c.1142C>A	p.Thr381Lys	NR	*AAAS* (NM_015665.6)	Homo	c.1432C>T	p.Arg478Ter	4.62e-5
8	4-026	*SPAST* (NM_014946)	Het	c.1356_1357insGGG	p.E452delinsEG	NR	*SPTBN2* (NM_006946.4)	Comp Het	c.6283C>T & c.5221C>T	p.Arg2095Trp & p.Arg1741Trp	2.89e-5 & 7.99e-6
9	4-047	*KIDINS220* (NM_020738.4)	Homo	c.2015C>T	p.Ser672Phe	NR	*SCN11A* (NM_014139.3)	Het	c.5116A>G	p.Met1706Val	3.98e-6
10	4-052	*SACS* (NM_014363.6) & (NM_001278055.2)	Comp Het	c.7817A>G & c.4459G>C	p.Asn2606Ser & p.Glu1487Gln	NR & 0.0000359	*KCNA2* (NM_004974.4)	Het	c.50G>A	p.Gly17Glu	NR
11	1-169	*ABCD1* (NM_000033.4)	Homo	c.346G>C	p.Gly116Arg	NR	*COL6A1* (NM_001848.3)	Het	c.1529A>G	p.Glu510Gly	NR

Discussion

Here we described the molecular landscape of the Canadian HSP cohort - one of the largest HSP cohorts that has been studied to date. We expanded the genetic spectrum of common and rare HSP subtypes. We reported a genetic diagnosis rate of 68.2%, higher than previously published studies (Orsucci *et al.*, 2014; Balicza *et al.*, 2016a; Kara *et al.*, 2016; Lynch *et al.*, 2016a; Schüle *et al.*, 2016a; Morais *et al.*, 2017a; Dong *et al.*, 2018b; Akçakaya *et al.*, 2020; Cui *et al.*, 2020) due to several reasons. First, we used exome-/genome-wide techniques. Second, we focused on the detection of all types of variants. Third, we screened all the genes related to diseases that share an overlapping zone with HSP. Fourth, by applying pathway enrichment and PPI analysis, we identified several candidate new genes. Fifth, we could provide some evidence for supporting non-Mendelian inheritance in HSP.

With the extensive use of ES in the genetic diagnosis of rare Mendelian disorders, CNVs and intronic variants are often underdiagnosed. Recently, a deep intronic *SPG7* allele which activates a cryptic splice site, was identified in a carrier of a missense *SPG7* allele (Verdura *et al.*, 2020). *SPG7* CNVs were tested in all heterozygous *SPG7* carriers in our cohort, and three patients with concurrent *SPG7* CNV and SNV were found. Interestingly we found a patient with *KIF1A* CNV highlighting GS as the most comprehensive method in HSP's genetic diagnosis. We further investigated the intronic and synonymous variants which have been previously confirmed as pathogenic. We reported the first patient with a homozygous deep intronic *POLR3A* variant, c.1909+22G>A (Gauquelin *et al.*, 2017; Minnerop *et al.*, 2017a; Minnerop *et al.*, 2019; Rydning *et al.*, 2019). This intronic variant has been indicated that could activate a cryptic splice site, resulting in a premature stop

codon leading to nonsense-mediated decay (NMD) (Rydning *et al.*, 2019). The disease caused by this intronic *POLR3A* variant in compound heterozygous form has been previously suggested to be categorized as an atypical POLR3-related disorder, rather than either HSP or ataxia (Gauquelin *et al.*, 2017).

Moreover, our findings from CanHSP cohort revealed that variants in causative genes can be either involved in predominant neurological symptoms that are the main clinical features of overlapping diseases such as ALS/PLS (e.g., *ALS2*, *SETX*), neuropathies (e.g., *DNM2*, *NEFH*, NEFL, *DYNC1H1*, *ATL3*), and ataxias (e.g. *SACS*, *VAMP1*, *AFG3L2*), or implicated in a phenotypes that only resemble HSPs, and do not manifest lower limb spasticity as a core feature. such as encephalopathies (e.g., *CSF1R*, *CACNA1E*, *DNM1L*), metabolic disorders (e.g., *MCOLN1*, *SLC2A1, SLC20A2, GALC, GCH1*) and rare syndromes (e.g., *TINF2*, *AAAS*, *LYST, PLA2G6*). We suggest that this list of genes should be added to molecular diagnostic pipelines for the diagnostic work-up of movement disorders. “Transport” and “Cell Projection” are shared pathways that were enriched between HSP and non-HSP genes detected in the current study (Supplementary Table 2.4). Our results along with the similar observation from other studies suggest implementing new nosology and reclassifying of all overlapping neurological disorders (Bajaj *et al.*, 2002; Eymard-Pierre *et al.*, 2002b; Zhan *et al.*, 2013; Balicza *et al.*, 2016b; Diomedi *et al.*, 2016b; Schüle *et al.*, 2016b; Synofzik *et al.*, 2016; Africa *et al.*, 2017; Elsayed *et al.*, 2017; Ozes *et al.*, 2017; Koh *et al.*, 2018; Nicita *et al.*, 2018; Simone *et al.*, 2018; Travaglini *et al.*, 2018; Elert-Dobkowska *et al.*, 2019b).

We recently provided evidence for non-Mendelian inheritance in HSP (Bis-Brewer *et al.*, 2020; Estiar *et al.*, 2021). In the present study, we provide additional evidence regarding

the role of digenic inheritance in HSP by identifying of 11 patients with potentially pathogenic variants within two related genes who presented with complex phenotypes. The phenotypic variability observed in our HSP patients within each subtype further supports the possibility of oligogenism in HSPs. These findings highlight the importance of applying exome/genome -wide analysis for genetic diagnosis rather than using a panel sequencing of several common HSP genes. Moreover, we detected four patients with homozygous *ATL1* and *SPAST* variants. The evidence for uniparental disomy in *FA2H* (SPG35) (Soehn *et al.*, 2016) as well as the previously reported discrete patterns in *SPG7* (Casari *et al.*, 1998), *ALDH18A1* (SPG9) (Coutelier *et al.*, 2015), *ERLIN2* (SPG18) (Rydning *et al.*, 2018) *KIF1A* (SPG30) (Lee *et al.*, 2015) and *KIF1C* (SPG58) (Dor *et al.*, 2014; Novarino *et al.*, 2014b) indicate that determining which genes to sequence in genetic counseling based on the mode of inheritance or clinical picture exclusively, could lead to misdiagnosis.

The genetic heterogeneity of HSPs render them a strong candidate for new gene discovery using a PPI network approach based on the known causative genes (Vavouraki *et al.*, 2021). We previously reported *SPTAN1*, *KPNA3*, *RBSN*, and *TCEAL1* as new genes in HSPs (Leveille *et al.*, 2019a; Estiar *et al.*, 2022; Hijazi H, 2022; Paul *et al.*, 2022). In the present study, we provided two more potential novel genes in HSPs, *AP5B1* and *CAPNS2*. AP5B1 and CAPNS2 are members of AP-5 and Calpain families respectively. The role of the members of both families in pathophysiology of HSP has been well-described. AP5B1 interacts with spastizin (SPG15) and spatacsin (SPG11) (Chang *et al.*, 2014b) and depletion of spatacsin or spastizin prominently destabilizes AP-5 (Hirst *et al.*, 2013a). The reduced level of AP5B1 was shown in fibroblasts from SPG11 and SPG15

patients as well as in cells with depletion of spatacsin or spastizin using siRNA (Chang *et al.*, 2014b). In addition, AP5B1, SPG11, and SPG15 have quite similar subcellular distributions, localizing to a late endosomal-lysosomal compartment, and are essential for long axons' survival and function (Hirst *et al.*, 2013a). Seemingly, the primary motor neurons are not the only affected neurons, and the AP-5/SPG11/SPG15 complex affects other neuronal types as well (Hirst *et al.*, 2018a), causing a complex HSP form.

Calpains are a family of highly conserved Ca^{2+}-dependent proteases (Mahaman *et al.*, 2018) that have been associated with different neurodegenerative disorders probably as a result of cellular Ca^{2+} homeostasis disturbances (Patzke and Tsai, 2002; Nixon, 2003; Mahaman *et al.*, 2018). CAPNS1 and CAPNS2 are two small subunits of Calpains and are distributed in dendrites and certain axons of rat brain respectively. The knockout of CAPNS1 is lethal in spite of the presumed continued expression of CAPNS2 (Friedrich *et al.*, 2004b; Bevers *et al.*, 2008). PPI analysis revealed that CAPNS2 interacts with partners of known HSP proteins, CAPN1 and SPTAN1, both of which have been reported as HSP genes by our team (Gan-Or *et al.*, 2016; Leveille *et al.*, 2019b).

The emergence of artificial intelligence-driven tools (Jumper *et al.*, 2021) to predict protein structures allowed us to make high-confidence predictions for the impact of HSP-associated variants in *CAPNS2* and *AP5B1*. In particular, the AP-5 β subunit had no significant sequence homology to other proteins in the Protein Data Bank (www.rcsb.org), which compromised our ability to predict its structure using previous homology modelling tools. By using the structure and global architecture of the related AP-2 complex, we were able to posit that the C-termini of the AP-5 β and ζ subunits interact and use that information to generate high confidence models for the interface where we find the

missense variant p.(Arg578Trp). However, these remain predictions, and experimental support would be required to confirm that these mutations indeed disrupt PPIs.

Here, we identified 164 variants that are not reported in gnomAD, and some of them occur in genes that were reported in either a single or few publications. Thus, it is crucial to expand the molecular and clinical spectrum of rare HSP subtypes. For instance, we reported the second HSP family with *C19orf12* variant (SPG43). Biallelic *MARS* variants has been reported in a single HSP case and confirmed by zebrafish model (Novarino *et al.*, 2014b). Two unrelated families in our cohort were identified carrying biallelic *MARS* variants (SPG70) with manifestations that are not reported before. Other examples of novel clinical findings are as follows: ataxia in *AP4S1*, hammer toes in *FARS2,* and motor delay and dysarthria in *ATP1A1*. Excluding the core features, sensory abnormalities, dysarthria, and ankle clonus were the most common sign in our patients. Overall, there was no specific sign or symptom that could predict the causative gene. However, there were several constellations of signs that were found often in specific subtypes, such as learning disability in SPG11 and cerebellar signs in SPG7.

Our study has some limitations. Although multiple *in silico* tools along with the previously published functional studies indicate the potential pathogenic role of the two candidate genes, additional reports of HSP patients with biallelic *AP5B1* and *CAPNS2* variants are required. We detected a large amount of rare intronic and synonymous variants that were not included in our list because their functional interpretation and further evidence are required.

In conclusion, our findings illustrate the heterogeneity of the diseases that are known to be a part of the umbrella term “HSP”. An accurate genetic diagnosis of HSP patients will

be essential not only for bespoke genotype-targeted therapy in the future, but also for ruling out similar diseases that have available treatments, such as metabolically-modifiable disorders, levodopa-responsive neurodegenerative disorders and the types of leukodystrophies that are managed with bone marrow transplantation.

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BRIDGING TEXT

We aimed to establish the genetic causes of all HSP cases in the cohort. Of the whole 375 families, the genetic diagnosis of 255 index cases was made by detecting variants within known HSP genes. Several HSP subtypes, such as SPG78, have only been described in a few studies and there is no well-described relevant clinical and genetic information. Moreover, several known HSP genes' variants detected in this study are also implicated in distinct phenotypes. For instance, *ATP13A2* is known to be involved in a very rare subtype of HSP, SPG78, as well as Kufor–Rakeb syndrome (KRS, OMIM 606693), a form of idiopathic early-onset Parkinson's disease. Interestingly, *ATP13A2* variants were reported in Neuronal Ceroid Lipofuscinosis (NCL) and amyotrophic lateral sclerosis (Farias *et al.*, 2011; Spataro *et al.*, 2019). The next chapter further elaborates on the genetic and clinical findings of SPG78. We reported three unrelated patients with homozygous *ATP13A2* mutations. Of these mutations, two have not been reported in the general population and, interestingly, one has already been reported in a patient with KRS (Eiberg *et al.*, 2012). From a clinical point of view, all our patients had psychiatric symptoms, reported previously in a single patient (Estrada-Cuzcano *et al.*, 2017). Additionally, one of the patients manifested seizure which has not been reported in SPG78.

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Clinical and genetic analysis of *ATP13A2* in hereditary spastic paraplegia expands the phenotype

Running title: *ATP13A2* in hereditary spastic paraplegia

Mehrdad A. Estiar[1,2], Etienne Leveille[3], Dan Spiegelman[2,4], Nicolas Dupre[5], Jean François Trempe[6,7], Guy A. Rouleau[1,2,4], Ziv Gan-Or[1,2,4]

[1] Department of Human Genetics, McGill University, Montréal, Quebec, Canada

[2] Montreal Neurological Institute and Hospital, McGill University, Montréal, Quebec, Canada

[3] Faculty of Medicine, McGill University, Montréal, Quebec, Canada

[4] Department of Neurology and Neurosurgery, McGill University, Montréal, Quebec, Canada

[5] Department of Medicine, Faculty of Medicine, Université Laval, Quebec City, QC, Canada.

[6] Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada.

[7] Centre for Structural Biology, McGill University, Montréal, Québec, Canada

Corresponding author:

Ziv Gan-Or

Department of Neurology and Neurosurgery

McGill University

1033 Pine Avenue, West,

Ludmer Pavilion, room 312

Montreal, QC, H3A 1A1,

Phone: +1-514-398-5845

Fax. +1-514 398-8248

Email: ziv.gan-or@mcgill.ca

Abstract

Background: Hereditary spastic paraplegias (HSP) are neurodegenerative disorders characterized by lower limb spasticity and weakness, with or without additional symptoms. Mutations in *ATP13A2*, known to cause Kufor–Rakeb syndrome (KRS), have been recently implicated in HSP.

Methods: Whole-exome sequencing was done in a Canada-wide HSP cohort.

Results: Three additional patients with homozygous *ATP13A2* mutations were identified, representing 0.7% of all HSP families. Spastic paraplegia was the predominant feature, all patients suffered from psychiatric symptoms, and one patient had developed seizures. Of the identified mutations, c.2126G>C;(p.[Arg709Thr]) is novel, c.2158G>T;(p.[Gly720Trp]) has not been reported in *ATP13A2*-related diseases, and c.2473_2474insAAdelC;p.[Leu825Asnfs*32]) has been previously reported in KRS but not in HSP. Structural analysis of the mutations suggested a disruptive effect, and enrichment analysis suggested the potential involvement of specific pathways.

Conclusion: Our study suggests that in HSP patients with psychiatric symptoms, ATP13A2 mutations should be suspected, especially if they also have extrapyramidal symptoms.

Keywords

ATP13A2, HSP, Neurodegeneration, Parkinsonism

1. INTRODUCTION

Hereditary spastic paraplegia (HSP) is a group of neurodegenerative disorders characterized by lower limb spasticity and weakness, with or without additional symptoms (Faber, Pereira, Martinez, França Jr, & Teive, 2017). Some HSP-related genes may be involved in other disorders in which spasticity is not among the main features, for example, *FA2H* (OMIM 611026) and *KIAA1840* (OMIM 610844) mutations may cause neurodegeneration with brain-iron accumulation and Charcot–Marie–Tooth (CMT) (Kruer et al., 2010; Montecchiani et al., 2015). Similarly, genes that are involved in other neurological disorders, such as *ALS2* (OMIM 205100) and *POLR3A* (OMIM 614258), were also implicated in HSP (Eymard-Pierre et al., 2002; Rydning et al., 2019).

One of the most interesting genes in the latter category is *ATP13A2* (OMIM 610513), which was initially implicated in Kufor–Rakeb syndrome (KRS, OMIM 606693), characterized by early onset parkinsonism, pyramidal tract degeneration, dementia, and cognitive dysfunction (Ramirez et al., 2006). Subsequently, *ATP13A2* mutations were reported in Neuronal Ceroid Lipofuscinosis (NCL) and amyotrophic lateral sclerosis (ALS; Farias et al., 2011; Spataro et al., 2019). *ATP13A2* mutations in HSP (SPG78, OMIM 617225) were first described in a consanguineous Pakistani family (Kara et al., 2016), followed by three reports on five more families (Erro, Picillo, Manara, Pellecchia, & Barone, 2019; Estrada-Cuzcano et al., 2017; van de Warrenburg et al., 2016). ATP13A2 encodes a lysosomal enzyme which serves as an inorganic cation transporter that regulates endolysosomal cargo sorting and neuronal integrity (Demirsoy et al., 2017; Ramonet et al., 2011).

Herein, we report three additional HSP patients from three different families with homozygous *ATP13A2* mutations. Long-term follow-up, genetic analysis, protein structure, and network analyses were done to explore the clinical and genetic spectrum of *ATP13A2*-related disease.

2. METHODS

2.1. Population

HSP patients (n = 696) from 431 families were recruited across Canada, and data on diagnosis, recruitment, and the cohort were previously published (Chrestian et al., 2017). Of those, 383 HSP genetically undiagnosed patients went through whole-exome sequencing (WES). All participants have signed an informed consent form and the study protocol was approved by the institutional review board.

2.2. Genetic analysis

Whole-exome capture, sequencing, alignment, annotation, and variant calling was performed as previously described (Chrestian et al., 2017). Nonsynonymous, frameshift, splice-site, and stop variants with allele frequencies <0.005 in the Exome Aggregation Consortium (ExAC) database were filtered-in, and segregation analysis was performed. The potential pathogenicity of variants was estimated based on their frequency of in gnomAD and ExAC, and by *in silico* tools: MutationTaster, combined annotation-

dependent depletion (CADD), genomic-evolutionary rate profiling (GERP++), sorting intolerant from tolerant (SIFT), and PolyPhen-2.

2.3. *In silico* analysis of *ATP13A2*

Genic intolerance of *ATP13A2* was assessed using the residual variation intolerance score (RVIS) tool. Pathways enrichment and interaction network were analyzed using GeneMANIA, g:Profiler, and STRING, and networks were visualized by Cytoscape. Clustal Omega program was used for protein sequence alignment of multiple species. A 3D atomic model of human ATP13A2 was built using the automated I-TASSER server. The steric clashes induced by each mutation were evaluated using the mutagenesis toolbox in PyMol v.2.2.0.

3. RESULTS

3.1. *ATP13A2* mutations are responsible for 0.7% of families with HSP in Canada and may affect the protein structure and function

Biallelic homozygous *ATP13A2* mutations were identified in three patients (representing 0.4% of HSP patients and 0.7% of families), including c.2473_2474insAAdelC;p.(Leu825Asnfs*32), c.2126G>C;p.(Arg709Thr), and c.2158G>T;p.(Gly720Trp). The p.(Leu825Asnfs*32) and p.(Arg709Thr) variants were not reported in gnomAD (https://gnomad.broadinstitute.org), and the p.(Gly720Trp) variant has a very low allele frequency of 0.000026 in Europeans in gnomAD. *ATP13A2* is highly

intolerable for functional genetic variations with an RVIS score of −1.16, putting it in the top 6.1% of intolerant human genes. Interaction network analysis (Figure1a) demonstrated that the ATP13A2 protein closely interacts with other HSP-related proteins. Pathway enrichment analysis of genes which are known to be involved in HSP, ALS, and Parkinsonism showed enrichment (FDR $p < .05$, Table S1) of genes involved in copper ion binding, vesicle-mediated transport cellular response to oxidative stress, among others. Both p.(Arg709Thr) and p.(Gly720Trp) destabilize the N-domain of ATP13A2 protein and are conserved (Figure 1b–e) and the p.(Leu825Asnfs*32) mutation deletes an entire segment at the C-terminal of the protein (Figure (Figure1c–e).1c–e). The distribution of the current and previously reported mutations in *ATP13A2* in HSP, KRS, ALS, and NCL (Park, Blair, & Sue, 2015; Spataro et al., 2019) is depicted in Figure1f.

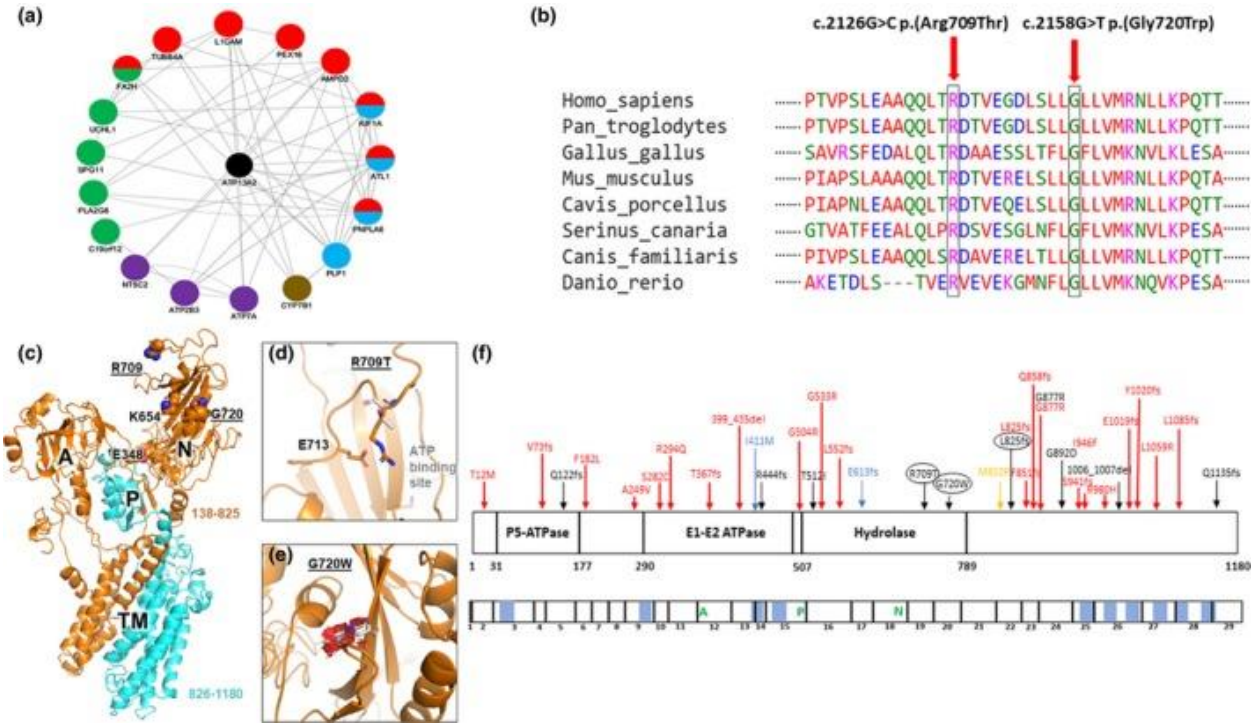

Figure 1

In silico analysis of ATP13A2. (a) Network analysis demonstrated that ATP13A2 is associated with other HSP-related proteins. Green: putative homologs are comentioned or coexpressed in other species, purple: shared protein domains, brown: genetic interactions, blue: colocalization, red: coexpression. (b) Conservation of the residues harboring missense mutations in the ATP13A2 protein. (c) Cartoon representation of human ATP13A2 a.a. 138–1180. The position of the cytosolic A-, P-, and N-domains, and transmembrane (TM) helices are indicated. Lys654 is an invariant lysine that interacts with the adenine ring of ATP prior to the g-phosphate transfer. Glu348 is the catalytic glutamate in the invariant TGE motif. The HSP mutation sites p.(Arg709Thr) and p.(Gly720Trp) are underlined. The segment consisting of a.a. 826–1180 (cyan) would be deleted in the p.(Leu825Asnfs*32) mutation. (d) Arg709 is located in the N-domain, on the opposite side of the ATP-binding site. The mutation p.(Arg709Thr) would result in the loss of a favorable electrostatic interaction, which would destabilize the N-domain. (e) Gly720 is located in the middle of a β-strand in the N-domain. The mutation p.(Gly720Trp) would create significant steric clashes (red), thus likely unfolding the N-domain. (f) Schematic representation of the location of *ATP13A2* mutations in HSP, ALS, KRS, and NCL patients reported so far (Park et al., 2015; Spataro et al., 2019). The top schematic represents the ATP13A2 protein. Functional domains, including the P-5 ATPase, E1-E2 ATPase, and hydrolase domains, are indicated with vertical lines. Mutations associated with HSP are indicated in black (mutations identified in this study are circled), ALS in blue, KRS in red, and NCL in yellow. The bottom schematic represents the cDNA of *ATP13A2*.

Exons are delineated with vertical line, and the location of the transmembrane domains are colored in blue

3.2. Clinical characteristics of HSP patients with *ATP13A2* mutations

Table 1 details the clinical characteristics of previously published *ATP13A2*-related HSP patients and the three patients identified in this study. The description of the patients below will detail only the main characteristics.

Table 1. Characteristics of HSP patients harboring *ATP13A2* mutations.

Nucleotide change	AA change	Inheritance	Clinical Signs	MRI	Age at Onset/Sex	Origin	Reference
c.3017_3019del	p.Leu1006_Leu1007del	H	Spastic quadriplegia, falls, cognitive decline, pes cavus, ataxia, bilateral divergent squints, nystagmus on lateral gaze, reduced upgaze, No parkinsonian features	Cerebral atrophy and subtle abnormalities of the basal ganglia	18/M	Pakistani	[9]
c.2675G>A	p.Gly892Asp	H	Spastic tetraplegia, cognitive decline, upgaze limitation, slow vertical saccades, mild Parkinsonism, gait abnormality, speech and swallowing difficulties, dysarthria, jerky eye movements, weakness and atrophy of the tongue, thoracic scoliosis, upper limb rigidity, bradykinesia on finger-tapping	Cerebral and cerebellar atrophy	11/M	Dutch	[10]
c.1535C>T	p.Thr512Ile	H	Lower limb spasticity, lower limb weakness, upper and lower limb hyperreflexia, Babinski sign, Oculomotor disturbance, dysarthria, limb ataxia, slight verbal memory deficit, surface sensation deficit, vibration deficit, mild cognitive impairment, cerebellar ataxia, and axonal motor and sensory polyneuropathy	Cerebellar > cortical atrophy, Periventricular white matter changes, ear of the lynx sign	30/M, 33/M, 30/M	Bulgarian	[18]
c.364C>T	p.Gln122Ter	H	Spastic paraplegia, Lower limb spasticity, lower limb weakness, upper and lower limb hyperreflexia, neurogenic bladder dysfunction, mild dysarthria, severe dementia, labile motivation, Oculomotor disturbance, limb ataxia, vertical supranuclear gaze palsy, urge incontinence, mixed axonal-demyelinating motor polyneuropathy	Cerebellar > cortical/mesencephalic atrophy, thin corpus callosum, hydrocephalus, periventricular white matter changes	36/F	Serbian	[18]
c.1330C>T/3403C>T	p.Arg444Ter/Gln1135Ter	C	Lower limb spasticity, upper and lower limb weakness, upper and lower limb hyperreflexia, Babinski signs, severe fronto-temporal dementia, aggression, acoustic hallucinations, Bradykinesia, resting tremor, oculomotor disturbance, dysarthria, limb ataxia, horizontal and vertical supranuclear gaze palsy, urge incontinence, divergent strabismus, mild axonal sensory, neuropathy	Cerebellar > cortical atrophy, ear of the lynx sign	32/F	Bosnian	[18]
c.2629G>A	p.Gly877Arg	H	Spastic gait, hyperreflexia, falls, bilateral adductor response of knee jerk, pyramidal hypertonia, questionable bradykinesia, dysdiadochokinesia, balance difficulties, ocular disturbances, slurred speech, mental retardation, Babinski signs, brisk reflexes, slightly increased axial and appendicular tone, mild parkinsonism	Generalized atrophy	31/M	Italy	[12]

c.2473_2474insAAdelC	p.(Leu825Asnfs*32)	H	Spastic paraplegia, lower extremity spasticity, lower extremity hyperreflexia, Babinski signs, spinocerebellar ataxia, dysarthria, falls, swallowing difficulty, cognitive decline, urinary complications, sensory abnormalities, mild upper extremity hyperreflexia, slow and ataxic saccades, dysdiadochokinesia, bucco-lingual dyskinesias, bradykinesia, action and Parkinson tremor, dysmetria, behavior problems	Diffuse cerebellar atrophy	31/F	Inuit Canadian	Present Study
c.2126G>C	p.Arg709Thr	H	Spastic paraplegia, lower extremity hyperreflexia, lower extremity spasticity, Babinski sign, upper extremity hyperreflexia, dysarthria, fine motor impairment, learning difficulty, mild intellectual disability, cognitive decline, atrophy, pes cavus, mild vibratory loss, seizures, mild nystagmus, saccadic pursuit, slow and ataxic saccades, fatigable right beating nystagmus, ataxic gait, delusions, hallucinations, bradykinesia, dysdiadochokinesia	Diffuse cerebral and cerebellar atrophy and hypoplasia of the corpus callosum.	25/M	Armenia-Lebanon	Present Study
c.2158G>T	p.Gly720Trp	H	Spastic paraplegia, lower extremity spasticity, lower extremity weakness, hearing difficulty, learning difficulty, falls, Babinski signs, ataxia, ankle clonus, dysarthria, dysphagia, saccadic pursuit, psychotic episodes, paranoid delirium, no parkinsonism	Cortical and cerebellar atrophy with signs of leukoencephalopathy in semioval centers, especially on the right side	29/M	French-Canadian	Present Study

Abbreviations – AA, Amino Acid; MRI, Magnetic Resonance Imaging; H, Homozygous; C, Compound Heterozygous; M, Male; F, Female.

3.2.1. Patient A

The patient, a 44-year-old woman of Inuit-Canadian origin, was initially evaluated at age 31 due to gait dysfunction. She was found to have bilateral lower extremity spasticity, weakness, hyperreflexia, nonsustained bilateral ankle clonus and speech difficulties. On evaluation at age 40, she was laughing excessively and seemingly had an inappropriate affect. Minor Parkinsonian tremor and action tremor were noted. At age 43, the patient was agitated and verbally and physically aggressive. Fine movements were decreased, and spinocerebellar ataxia and prominent spastic paraplegia were present. Some of her parkinsonian symptoms may be attributed to her treatment with haloperidol. Brain and spine MRI demonstrated diffuse cerebellar atrophy and normal spine (Figure2a). WES revealed a p.(Leu825Asnfs*32) mutation which results in a truncated peptide of 857 a.a and deletion of six C-terminally located transmembrane alpha-helixes. The mutation has not been reported in gnomAD and ExAC, but was previously reported (also as homozygous) in a patient with KRS from a Greenlandic Inuit family (Eiberg et al., 2012). As our patient is of Inuit-Canadian family, this might suggest that this is an old, founder Inuit mutation.

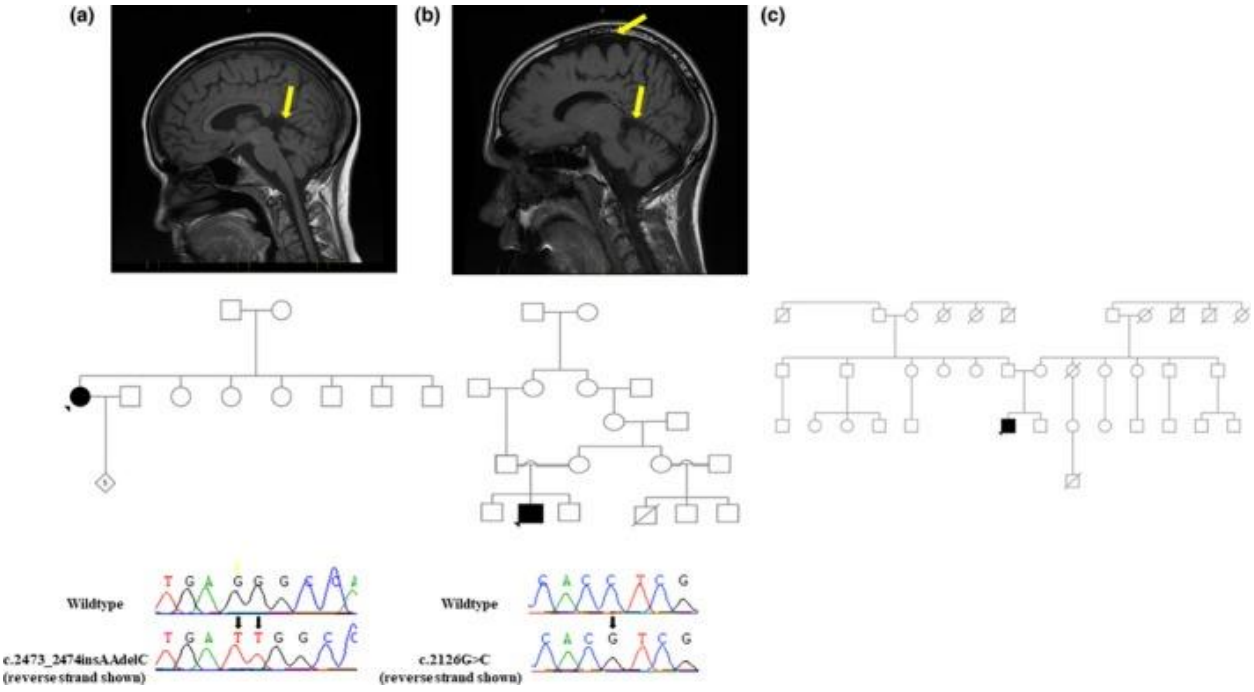

Figure 2

MRI images, pedigrees, and Sanger sequencing chromatograms. (a) Patient A's MRI showed diffuse cerebellar atrophy (arrow). (b) Patient B's MRI showed moderate diffuse cerebral and cerebellar atrophy (arrows). (c) MRI and DNA for sanger sequencing were not available for patient C.

3.2.2. Patient B

During childhood, this patient from an Armenian-Lebanese consanguineous family had impairment of fine motor movements, and experienced learning difficulties and delayed mental development noticeable at the age of 6 years. On evaluation at age 18, the patient presented with increased muscle tone especially in the lower extremities and gait was spastic. A Levodopa trial did not result in any improvement. At age 24, the patient had a

seizure for the first time. MRI demonstrated moderate diffuse cerebral and cerebellar atrophy (Figure2b). EEG demonstrated mild, slow biposterior dysfunction but without epileptiform patterns. On evaluation at the age of 31 years, the patient started to develop ideas of reference and delusions. WES was performed and identified a novel homozygous missense *ATP13A2* mutation, p.(Arg709Thr), in exon 19 within the hydrolase domain. The mutation is predicted to be deleterious by CADD (25), Polyphen-2 (0.99), MutationTaster (1), and was located in a highly conserved amino acid with GERP++ score of 5.

3.2.3. Patient C

At age 6, after normal development, this male patient of French-Canadian origin was reported to have learning difficulties that became more pronounced through high school. At age 12, the patient started abusing alcohol and drugs, and throughout his teenage years he had two psychotic episodes and paranoid delusions. On evaluation at age 32, the patient had presented with spasticity and ataxia, spastic and mildly magnetic gait with frequent falls. Brain MRI done at the age of 29 showed cortical and cerebellar atrophy (images are not available). Metabolic workup, EEG, EMG, nerve conduction studies, and an abdominal ultrasound were normal. Clinical WES identified a homozygous *ATP13A2* missense mutation in exon 20, p.(Gly720Trp), predicted to be deleterious by SIFT (0), Polyphen-2 (1), and CADD (31).

4. DISCUSSION

We describe three unrelated patients with predominant spastic paraplegia features, harboring homozygous *ATP13A2* mutations that are either novel or were not previously

reported in HSP. *ATP13A2*-HSP is rare, responsible for 0.4% of all HSP patients and 0.7% of all HSP families in CanHSP. Interestingly, all three patients suffered from psychiatric symptoms, which were previously reported in only one SPG78 patient (Estrada-Cuzcano et al., 2017). One of the patients has developed seizures, which have not been previously reported in SPG78. Mild extrapyramidal symptoms/signs were present in patient A and bradykinesia in patient B. MRI in all three patients demonstrated cerebellar and/or cerebral atrophy, consistent with previous reports on *ATP13A2*-HSP (Table11).

Interestingly, the p.(Leu825Asnfs*32) mutation in patient A resulted in HSP-predominant phenotype, while in previous patients reported with the same mutation it was Parkinsonism-dominant phenotype (Eiberg et al., 2012). This may suggest that the clinical presentation may be affected by other genetic and/or environmental factors. We also identified a novel missense *ATP13A2* variant, p.(Arg709Thr) in a highly conserved amino acid located within the hydrolase domain which is critical for the catalytic activity of *ATP13A2*. Furthermore, this variant is affecting the last nucleotide of the exon, which may also affect splicing and possibly result in nonsense mediated decay. This possibility needs to be studied preferably in neuronal models with the variant. The mutation in patient C, p.(Gly720Trp) changes Glycine to Tryptophan at codon 720, which could unfold the N-domain of ATP13A2 (Figure1e). Our pathway enrichment analysis may suggest that copper ion binding is involved in the pathogenesis of specific forms of HSP, and further studies are required to examine this possibility.

This study has several limitations. Since DNA was not available for segregation analysis, and since our genetic data include only in silico prediction tools and structural models, we could not prove with full confidence that the detected variants in *ATP13A2* are disease causing. However, one of the mutations was previously described in a patient, and it is unlikely that by chance alone two extremely rare biallelic variants in a gene that is already known as disease causing will be found in two HSP patients. Therefore, it is probable that these variants are disease causing. An additional limitation is the lack of available DNA for patient C, therefore the variant reported by the clinical lab could not be independently confirmed.

Our study expands the genetic and phenotypic spectrum of *ATP13A2*-related HSP. The different phenotypes observed in carriers of *ATP13A2* mutations imply that genetic and nongenetic modifiers exist. Our findings may also suggest that in HSP patients with psychiatric symptoms, mutations in *ATP13A2* should be suspected, especially if mild parkinsonian symptoms are also present.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

MAE, GAR, and ZG-O conceived the study design, MAE, EL, DS, ND, and JFT performed data analysis MAE and ZG-O wrote the paper. All authors have read, edited, and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

All data reported here are available upon request.

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BRIDGING TEXT

Thus far, we presented the genetic landscape for HSPs in our Canadian cohort and provided more details regarding one of the rare HSP subtypes. We applied several strategies to establish the genetic diagnosis for as many patients as possible. For example, the combined molecular diagnostic approaches were utilized. In addition, exome and genome wide analysis allowed us to screen all the genes associated with HSP-like conditions as well as the candidate genes, and subsequently analyze the proteins corresponding to these genes closely interact with known HSP molecules. It is likely that the existing huge genetic diagnostic gap in HSP partially comes from the occurrence of variants within the genes that are not associated with HSP, or not associated with any disease at the time begin. Two new genes, *AP5B1* and *CAPNS2*, were introduced in chapter 2 that were detected in a single family in our cohort. In the next chapter, we describe *SPTAN1* as a new gene in HSP. Two families with compound heterozygous *SPTAN1* variants were identified who presented with pure-HSP. Interestingly, one of the variants is the same in both French-Canadian families.

CHAPTER 4: MANUSCRIPT 3: Published in *Journal of Human Genetics* 64.11 (2019): 1145-1151.

Title page

SPTAN1 variants as a potential cause for autosomal recessive hereditary spastic paraplegia

Running title: *SPTAN1* variants in hereditary spastic paraplegia

Etienne Leveille,[1,*] Mehrdad A. Estiar,[2,3,*] Lynne Krohn,[2] Dan Spiegelman,[3,4] Alexandre Dionne-Laporte,[3,4] Nicolas Dupré,[5,6] Jean François Trempe,[7,8] Guy A. Rouleau,[2,3,4] Ziv Gan-Or,[2,3,4]

Author affiliations

[1]Faculty of Medicine, McGill University, Montréal, Quebec, Canada

[2]Department of Human Genetics, McGill University, Montréal, Quebec, Canada

[3]Montreal Neurological Institute and Hospital, McGill University, Montréal, Quebec, Canada

[4]Department of Neurology and Neurosurgery, McGill University, Montréal, Quebec, Canada

[5]Division of Neurosciences, CHU de Québec, Université Laval, Québec City, QC, Canada

[6]Department of Medicine, Faculty of Medicine, Université Laval, Québec City, QC, Canada

[7]Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada.

[8]Centre for Structural Biology, McGill University, Montréal, Québec, Canada

*These authors contributed equally to the work

Correspondence to:

Guy A. Rouleau,

Director, Montréal Neurological Institute and hospital,

Address: 3801, University Street, Office 636

Montréal, Québec H3A 2B4

Phone: +1-514-398-2690,

Fax. +1-514 398-8248

Email: guy.rouleau@mcgill.ca

Ziv Gan-Or

Department of Neurology and Neurosurgery

McGill University

1033 Pine Avenue, West,

Ludmer Pavilion, room 312

Montreal, QC, H3A 1A1,

Phone: +1-514-398-5845

Fax. +1-514 398-8248

Email: ziv.gan-or@mcgill.ca

Abstract

More than 80 known or suspected genes/loci have been reported to be involved in hereditary spastic paraplegia (HSP). Genetic and clinical overlap have been reported between HSP and other neurological condition, yet about 50% of HSP patients remain genetically undiagnosed. To identify novel genes involved in HSP, we performed a genetic analysis of 383 HSP patients from 289 families with HSP. Two patients with biallelic *SPTAN1* variants were identified; one carried the c.2572G>T p.(Ala858Ser) and c.4283C>G p.(Ala1428Gly) variants, and the second also carried the c.2572G>T p.(Ala858Ser) variant, and an additional variant, c.6990G>C p.(Met2330Ile). In silico predictive and structural analyses suggested that these variants are likely to be deleterious. *SPTAN1* was highly intolerant for functional variants (in the top 0.31% of intolerant genes) with much lower observed vs. expected number of loss-of-function variants (8 vs. 142.7, $p < 5 \times 10-15$). Using public databases of animal models and previously published data, we have found previously described zebrafish, mouse, and rat animal models of *SPTAN1* deficiency, all consistently showing axonal degeneration, fitting the pathological features of HSP in humans. This study expands the phenotype of *SPTAN1* mutations, which at the heterozygous state, when occurred de novo, may cause early infantile epileptic encephalopathy-5 (EIEE5). Our results further suggest that *SPTAN1* may cause autosomal recessive HSP, and that it should be included in genetic screening panels for genetically undiagnosed HSP patients.

Introduction

Hereditary spastic paraplegia (HSP) is a group of rare hereditary neurodegenerative motor neuron disorders, characterized by symmetrical spasticity and weakness of the lower extremities. Based on the absence or presence of additional neurological symptoms, HSP can be classified as pure or complex. Pure HSP may also be characterized by lower/upper limb hyperreflexia and extensor plantar responses, with or without urinary dysfunction; any additional neurological signs or symptoms will define complex HSP. The main pathological hallmark of HSP is thought to be degeneration of axons within the descending corticospinal tract and ascending sensory fibers [1].

To date, there are more than 80 genes or loci suggested to be involved in HSP, which include all patterns of Mendelian and non-Mendelian inheritance [2,3,4]. However, between 50 and 60% of HSP patients remain genetically undiagnosed [5], suggesting that numerous genes involved in HSP are yet to be discovered. While HSP is defined as an independent clinical entity, both clinical and genetic overlap exist between HSP and other disorders such as ataxia, seizure disorders, and others [4, 6]. This is further exemplified by the identification of clinically diagnosed HSP patients, who have variants in genes typically causing other disorders, such as *POLR3A* [7] (OMIM 607694), *SLC2A1* [8] (OMIM 612126), *AAAS* [9] (OMIM 231550), and *ATP13A2* [10] (OMIM 617225).

In the current study, we performed a thorough genetic analysis in one of the world's largest cohorts of HSP, CanHSP [11], to identify novel genes involved in HSP. Based on our findings, including *in silico* analysis and previously published zebrafish, mouse and

rat models, we report that biallelic *SPTAN1* (OMIM 613477) variants may cause autosomal recessive pure HSP.

Materials and methods

Population

A total of 696 HSP patients from 431 families were recruited from eight major medical centers across Canada (Montreal, Quebec, Toronto, Ottawa, Calgary, Edmonton, Hamilton, and Vancouver) from February 1, 2012 to December 31, 2018, and data on diagnosis, recruitment and the cohort were previously published [11]. All participants signed informed consent forms, and the study protocol was approved by the institutional review board. Of these, 383 HSP patients from 289 families went through whole exome sequencing (WES) and were included in the analysis. In addition, 251 unrelated healthy individuals that went through WES in our lab served as controls.

Genetics analysis

Whole exome capture, sequencing, alignment, annotation, and variant calling was done as previously described [11]. For detection of autosomal recessive genes, filtering was applied to select variants with a frequency < 0.005 in the exome aggregation consortium (ExAC) database. Nonsynonymous, frameshift and stop -gain variants that segregated with the disease were filtered in. Evaluation of the pathogenic potential of the variants was based on their frequency of in ExAC and genome aggregation database (gnomAD), and by prediction and conservation tools: combined annotation dependent depletion (CADD), MutationTaster, and genomic evolutionary rate profiling (GERP++). Segregation

analysis was performed and lists of potential disease-causing variants were generated for each family. Subsequently, we generated a list of all genes known or suspected to be involved in HSP, as well as genes that are associated with diseases that can mimic HSP or may have spasticity as one of their symptoms (a total of 660 genes, Supplementary Table 1), and cross-examined the filtered -in segregating variants with this list. Validation and further segregation of the suspected pathogenic variants were performed using Sanger sequencing, primer sequences are available upon request. WES data from 251 unrelated healthy individuals of European descent, without any family history of neurological diseases, were screened for presence of missense or LoF variants in *SPTAN1*.

In silico analysis of *SPTAN1* and the identified variants

Genic intolerance of *SPTAN1* was assessed using the residual variation intolerance score (RVIS) tool [12], and observed vs. expected variant frequencies in gnomAD. To examine the potential effect of these variants on SPTAN1 function and structure, SWISS-MODEL was used to generate homology models of human spectrin repeats [13]. Human α-spectrin repeats 7–8 were modelled using the chicken α-spectrin repeats 15–16 (pdb 1u5p). Human α-spectrin repeat 20 and EF hands 1–2 were modelled using the structure of human muscle α-actinin-2 (pdb 4d1e). Coordinates of human α-spectrin repeats 12–13 were derived from the original crystal structure (pdb 3fb2, a.a. 1337–1544). Structural analysis of the variants and images were generated using PyMOL v.2.2.0. Pathways and protein–protein interaction network were analyzed using WebGestalt [14] and STRING [15].

Literature and database search for animal and cellular SPTAN1 models

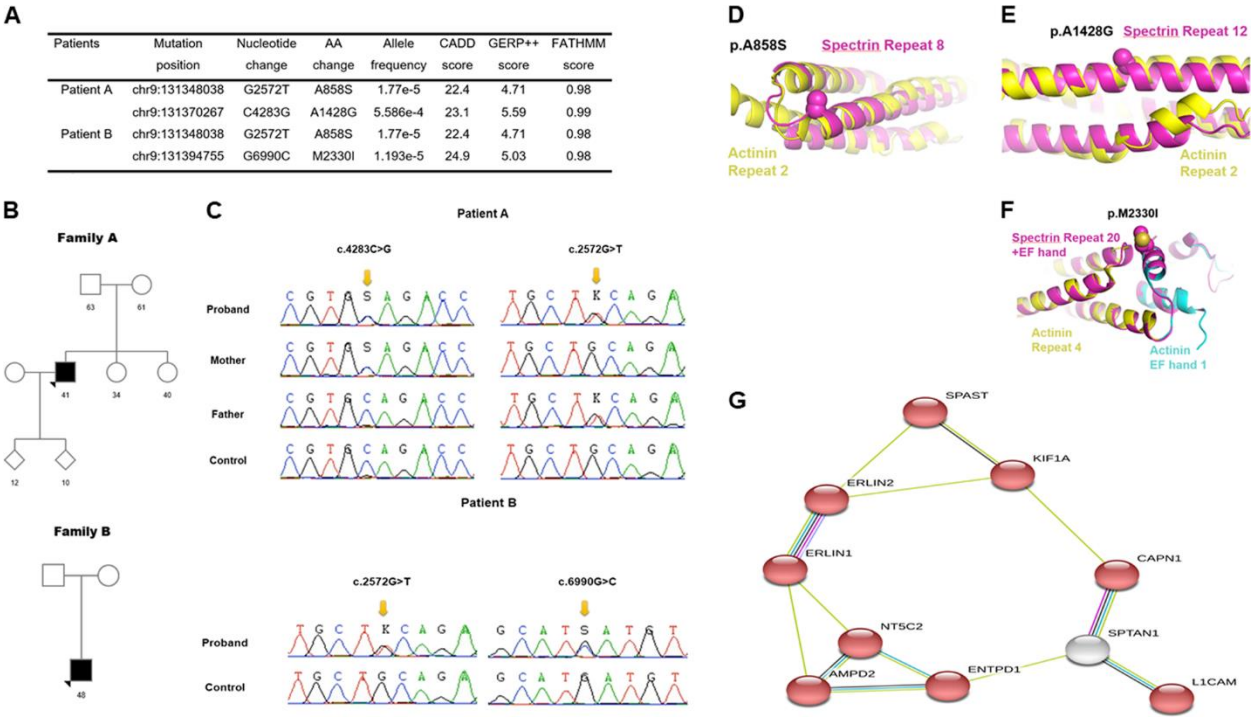
Since *SPTAN1* was already implicated in other disorders, we examined the literature and publicly available databases to examine whether animal and cellular models already exist that can explain HSP in the studied patients. PubMed was searched using the search words “SPTAN1”, “spectrin alpha”, “α-II Spectrin”, “Nonerythrocytic 1”, with the word “model”, and all relevant abstracts were screened to identify relevant animal or cellular models. The public databases Mouse Phenome Database, the International Mouse Phenotyping Consortium, Rat Genome Database, Mouse Genome Informatics, the Zebrafish Information Network, and Alliance of Genome Resources were accessed.

Results

Biallelic *SPTAN1* variants may explain HSP in two families

Following quality control and filtration, biallelic variants in *SPTAN1* remained the only explanation for HSP in two individuals. In proband A, two *SPTAN1* variants were identified, c.2572G>T p.(Ala858Ser) and c.4283C>G p.(Ala1428Gly) (NM_003127), and segregation was further confirmed by Sanger sequencing of the parents. Proband B also carried one of these variants, c.2572G>T p.(Ala858Ser), and an additional variant, c.6990G>C p.(Met2330Ile) (NM_003127). DNA from other family members of patient B was not available for segregation analysis. All three variants were predicted to be deleterious by CADD and MutationTaster, and were located in highly conserved amino acids with all GERP++ scores > 4.7 (Fig. 1a–c). According to the American College of Medical Genetics criteria and using VarSome tool, these variants were classified as “variants of uncertain significance”. One of these variants, p.(Met2330Ile), was not

reported in gnomAD and the other two variants are rare and no homozygous carriers of either were reported. Furthermore, in 251 unrelated healthy controls, no biallelic carriers of missense, splice site, or nonsense mutations were identified.



Patients	Mutation position	Nucleotide change	AA change	Allele frequency	CADD score	GERP++ score	FATHMM score
Patient A	chr9:131348038	G2572T	A858S	1.77e-5	22.4	4.71	0.98
	chr9:131370267	C4283G	A1428G	5.586e-4	23.1	5.59	0.99
Patient B	chr9:131348038	G2572T	A858S	1.77e-5	22.4	4.71	0.98
	chr9:131394755	G6990C	M2330I	1.193e-5	24.9	5.03	0.98

Fig. 1 *SPTAN1* variants, in silico analysis. **a** Four *SPTAN1* variants were identified in patients A and B. One of these variants is the same in both patients. All variants are rare enough in gnomAD to be disease causing in a recessive manner, are highly conserved and predicted to be deleterious. CADD combined annotation dependent depletion, GERP++ genomic evolutionary rate profiling, FATHMM functional analysis through hidden Markov models. **b** Pedigrees of the two HSP probands harbouring the *SPTAN1* variants. Open symbol: unaffected; filled symbol: affected; arrow: proband. **c** Sanger sequencing traces confirming the two variants (c.4283C>G and c.2572G>T) in patient A.

c.4283C>G p.(Ala1428Gly) and c.2572G>T p.(Ala858Ser) variants were observed in the mother and father of patient A, respectively. NM_003127:c.[2572G>T]; [6990G>C] variants were confirmed in patient B, but segregation analysis could not be performed as DNA was not available. The healthy controls did not present any of the variants. Genomic positions are based on the GRCh37/hg19 genome assembly. **d** Model of human α-spectrin repeat 8 (a.a. 785–887) derived from the structure of repeat 15 from chicken α-spectrin (pdb 1u5p). The side chain of Ala858 is shown as spheres. The model is superposed on the structure of α-actinin repeat 2 (pdb 4d1e) to highlight the proximity of the variant site to the preceding repeat. **e** Structure of human α-spectrin repeat 12 (pdb 3fb2, a.a. 1337–1544). The side chain of Ala1428 is shown as spheres. The model is superposed on the structure of α-actinin repeat 2 to highlight the kink that might be introduced in the helix harboring the variant site. **f** Model of human α-spectrin repeat 20 and EF hands 1–2 (a.a. 2206–2400) derived from the structure of α-actinin repeat 4 and EF hands 1-2 (pdb 4d1e). The position of Met2330 is shown as sphere and makes contact with repeat 20. **g** Protein–protein interaction network of SPTAN1 using STRING. The interaction partners of SPTAN1 are known to cause spastic paraplegia in this figure. SPTAN1 protein closely interacts with CAPN1 (SPG76), L1CAM (X-linked SPG), and ENTPD1 (SPG64)

The α-spectrin protein, encoded by *SPTAN1*, is 2472 a.a. long and consists of eight spectrin repeats, followed by an SH3 domain, 12 additional repeats, and 3 calcium-binding EF hands [16]. While there are structural coordinates for fragments of α-spectrin [17], they do not contain all the variants. We thus performed homology modelling of missing fragments, using crystal structures with the highest sequence identity to the target, in order to predict the effect of missense variants on the structure and function of the protein. The c.2572G>T p.(Ala858Ser) variant is located in the eighth spectrin repeat, which we modelled on the chicken α-spectrin-repeats 15–16 [18]. This variant would not create any steric clash but might affect the conformation of the loop that precedes Ala858 (Fig. 1d). Comparison with the structure of full-length α-actinin [19], which is homologous to spectrin, shows that this loop is in proximity with the preceding repeat. Therefore, the variant c.2572G>T p.(Ala858Ser) might affect the rigidity of the interaction between α-spectrin-repeats 7 and 8. The variant c.4283C>G p.(Ala1428Gly) is located in the 12th spectrin repeat. The structure of human α-spectrin-repeats 12–13 has been determined by X-ray crystallography at 2.3 Å resolution (NSGC target HR5563a; https://doi.org/10.2210/pdb3FB2/pdb). The variant site is located in the middle of a long helix, and variant to a glycine might lower the rigidity of the helix in this position (Fig. 1e). Superposition with the structure of α-actinin reveals a kink in the helix at this position, suggesting this is a pivotal point in the structure. The variant c.6990G>C p.(Met2330Ile) is found in the first EF hand, a calcium-binding domain that regulates contacts with F-actin in response to calcium [20, 21]. The variant is very close to the spectrin-repeat 20, and thus we modelled both repeat 20 and EF hands 1–2 using the structure of α-actinin

[19]. The side chain of Met2330 interacts with repeat 20, and variant to an isoleucine might destabilize this interaction and “uncouple” these two domains (Fig. 1f).

SPTAN1 is highly intolerable for functional genetic variations with an RVIS score of −3.53, putting it on the top 0.31% of human genes in terms of genic intolerance. Comparing the expected number of loss-of-function (LoF) variants (n = 142.7) vs. the observed number of LoF variants (n = 8) in gnomAD, yielded a likelihood of 1.00 for intolerability with $p < 5 \times 10^{-15}$. Comparing observed vs. expected rare missense variants (allele frequencies < 0.001), highly significant z-scores were reported in ExAC (z = 6.8, p < 0.00001) and gnomAD (z = 5.8, p < 0.00001), further demonstrating lack of tolerability of *SPTAN1* for rare missense variants. Protein interaction analysis using STRING, including all the known HSP genes and *SPTAN1* suggested that *SPTAN1* interacts directly or indirectly with multiple HSP genes (Fig. 1g). Pathway-based analysis revealed SPTAN1 role in “intracellular transport”, “axon development”, and “axon” pathways, closely interacting with other HSP-related genes (Supplementary Table 2).

Clinical characteristics of HSP patients with *SPTAN1* variants

Both patients presented with pure HSP, and their clinical characteristics together with other findings are detailed in Table 1. Onset ages were 33 and 15 in patients A and B, respectively, with a slowly progressive course, involving the lower limbs, with minor involvement of the urinary bladder in patient A, and hyperreflexia of the upper limbs in both patients. SPATAX-EUROSPA disability stage was 0 (no functional handicap) and 3 (moderate, unable to run, and limited walking without aid) in patients A and B, respectively. In both, brain and spine MRI were normal, with no seizures or intellectual

disability. Phenotypically, both patients cannot be distinguished from other patients with pure HSP.

Table 1. Demographic and Clinical characteristics of the two HSP patients.

Characteristics	Patient A	Patient B
General Information		
Gender	Male	Male
Age at Evaluation	41	48
Age of Onset	33	15
Consanguinity	No	No
Mode of Inheritance	Recessive	Recessive
Ancestral Background	French Canadian	French Canadian
Core Symptoms		
Low Extremity Weakness	-	-
Low Extremity Spasticity	+	+
Low Extremity Hyperreflexia	+	+
Extensor Plantar Responses	-	+
Abnormal Bladder Function	+	-
Ankle Clonus	-	+
Other Symptoms		
Cognitive Deficits	-	-
Retinopathy or Optic Atrophy	-	-
Extraocular Movement	+	-
Deafness	-	-
Swallowing Difficulties	-	-
Dysarthria	-	-
Upper Extremity Weakness	-	-
Upper Extremity Hyperreflexia	+	+
Amyotrophy	-	-
Sensory Abnormalities	-	+
Peripheral Neuropathy	-	-
Pes Cavus	-	-
Ataxic Gait	-	-
Upper Extremity Ataxia	-	-
Upper Extremity Intent Tremor	-	-
Lower Extremity Ataxia	-	-
Lower Extremity Intent Tremor	-	-
Seizures	-	-
Skeletal Abnormalities	-	-
Myoclonus	-	-
Tests		

Brain MRI	Normal	Normal
Total Spine MRI	Normal	Normal

Previously published *SPTAN1* models are highly relevant for HSP

A zebrafish model with homozygous nonsense *SPTAN1* variants was reported to disrupt clustering of sodium channels around the nodes of Ranvier in CNS and PNS myelinated axons. In this model, α-II Spectrin, encoded by *SPTAN1*, was shown to be detrimental for the assembly of these nodes [22]. Similar results were observed in a mouse model [23] where loss of α-II Spectrin also disrupted the assembly of nodes of Ranvier and thus the ability of axons to rapidly propagate action potentials using salutatory conduction. Importantly, loss of α-II Spectrin led to degeneration of large myelinated axons in this model, which is similar to the pathology observed in HSP. Furthermore, these mice demonstrating hindlimb clasping reflex [23], which is a measure of neurodegeneration relevant to neurological disorders such as spasticity/ataxia [24]. In a rat model of loss of α-II Spectrin, axon development was impaired, demonstrating disruption of the formation of axon initial segment, as well as decreased and disorganized arborization [25].

Discussion

This is the first report suggesting that biallelic *SPTAN1* variants may lead to pure autosomal recessive HSP, further expanding the phenotypic spectrum associated with *SPTAN1* variants. However, additional patients with biallelic *SPTAN1* variants need to be identified to conclusively determine that *SPTAN1* variants cause recessive HSP. However, the fact that in the two affected individuals the same variant was identified,

c.2572G>T p.(Ala858Ser), support the pathogenicity of this variant and this gene in HSP. This was further supported by the structural analysis of these *SPTAN1* variants, as well as by the genic intolerance analysis and the previously reported animal models that have features that are similar to those seen in HSP. While we acknowledge as a limitation of the current study that only two patients are reported here, our data support the pathogenicity of the identified *SPTAN1* variants. Another limitation is that segregation analysis could not be performed for patient B, as DNA from the parents or other relatives was not available.

Dominant de novo *SPTAN1* mutations may cause early infantile epileptic encephalopathy-5 (EIEE5), with a suggested dominant-negative effect of the mutations [25]. In contrast to the patients described here, patients with EIEE5 have a much more severe disease, with MRI findings, which include diffuse hypomyelination and widespread brain atrophy which affects their cortex, corpus callosum, brainstem, and cerebellum [26, 27]. These phenotypic differences may suggest that the de novo mutations are severe, while the variants described in the current study are probably tolerable in heterozygous carriers, as evident by their rare presence in ExAC and gnomAD, and with an additional mutation may lead to a milder phenotype of pure HSP. Similar instances exist in other forms of HSP. For example, mutations in PLP1 may lead to a pure form of HSP, but also to severe forms of Pelizaeus–Merzbacher disease [28]. Of note, only a few EIEE5 patients with spasticity were reported, and it was also more severe than in the patients described here, accompanied by many other symptoms [27]. This may suggest that heterozygous *SPTAN1* mutations may also lead to HSP, considering that other HSP-

related genes were also reported to cause HSP in both autosomal recessive and autosomal dominant manners (e.g., *ATL1*, *ALDH18A1*, and *SPG7*) [29,30,31,32].

The animal models that were previously published [22, 23] provide an additional potential link to HSP, as some of the phenotypic consequences of loss of α-II Spectrin in these models, mainly the axonal neurodegeneration [23], are very similar to those observed in HSP [33]. Mutations in other HSP-related genes, such as *PLP1* [28], and *FA2H* [34], also affect myelination, as was seen in the aforementioned models. Therefore, the current findings further imply that disturbances in myelination may also be important in the pathogenesis of HSP.

Based on our findings, *SPTAN1* should be considered in HSP and included in sequencing panels for HSP. *SPTAN1* variants are likely to be rare in HSP, as in our cohort only 2/696 patients (0.3%) carry these variants. Nevertheless, additional studies in other populations are required to confirm the role of *SPTAN1* in HSP, and functional studies are needed to determine the exact mechanism by which *SPTAN1* variants cause HSP.

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BRIDGING TEXT

We were able to decipher the genetic diagnosis of two more HSP families in our cohort by introducing a new gene, *SPTAN1,* in HSP. Following our study, a novel homozygous *SPTAN1* mutation was reported in a patient with complex-HSP (Xie *et al.*, 2022). In a recent study, *de novo* and dominantly inherited *SPTAN1* mutations were also reported. In this study, analyzing large NGS datasets revealed 14 probands with five novel heterozygous *SPTAN1* variants who presented with ataxia or/and spastic paraplegia (Van de Vondel *et al.*, 2022). Reports of additional patients with consistent phenotype, as well as some functional evidence (Voas *et al.*, 2007; Guyenet *et al.*, 2010; Huang *et al.*, 2017; Wang *et al.*, 2018), strongly support the role of SPTAN1 in HSP. Mixed models of inheritance have already been reported in *KIF1A*, *ALDH18A1*, and *REEP2* (OMIM). Given the large sample of patients, we sought to deepen in a controversial aspect related to the mode of inheritance in SPG7. The most remarkable findings of the study in the next chapter are the detection of a frequency of heterozygous carriers of mutations in *SPG7* higher than in control population as well as the coexistence in some of these subjects of mutations in other SPG genes or rare variants in genes that code for proteins that interact with SPG7, this raising the possibility of a digenic inheritance or epistasis as an alternative in some cases to the AR inheritance attributed to this disease. Among digenic cases, there are strong functional evidence regarding the phenotype caused by concurrent *AFG3L2* and *SPG7* mutations (Martinelli *et al.*, 2009; Magri *et al.*, 2018). This research provides relevant data to shed light on some aspects related to the inheritance of SPG7, while recognizing at the same time the possibility that heterozygous cases might just reflect limitations of exome sequencing for detecting deep intronic mutations or CNVs.

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Evidence for Non-Mendelian Inheritance in Spastic Paraplegia 7

Mehrdad A Estiar[1,2], EricYu[1,2], Ikhlass Haj Salem[3], Jay Ross[1,2], Kheireddin Mufti[1,2], Fulya Akçimen[1,2], Etienne Leveille[4], Dan Spiegelman[2], Jennifer A Ruskey[2], Farnaz Asayesh[2], Alain Dagher[2], Grace Yoon[5], Mark Tornopolsky[6], Kym Boycott[7], Nicolas Dupre[8,9], Patrick A Dion[1,2,10], Oksana Suchowersky[11], Jean-Francois Trempe[12,13], Guy A Rouleau[1,2,10], Ziv Gan-Or[1,2,10]

1. Department of Human Genetics, McGill University, Montréal, Quebec, Canada

2. The Neuro (Montreal Neurological Institute-Hospital), McGill University, Montréal, Quebec, Canada

3. Faculté de Médecine, Université Laval, Québec (QC), Canada

4. Faculty of Medicine, McGill University, Montréal, QC, Canada

5. Divisions of Neurology and Clinical and Metabolic Genetics, Department of Paediatrics, University of Toronto, The Hospital for Sick Children, Toronto, Canada

6. Department of Pediatrics and Medicine, McMaster University, Hamilton, Canada

7. Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, Ontario, Canada

8. Neuroscience Axis, CHU de Québec, Université Laval, Québec City, Québec, Canada

9. Department of Medicine, Faculty of Medicine, Université Laval, Québec City, Québec, Canada

10. Department of Neurology and Neurosurgery, McGill University, Montréal, Québec, Canada

11. Departments of Medicine (Neurology) and Medical Genetics, University of Alberta, Edmonton, Alberta, Canada

12. Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada

13. Centre de Recherche en Biologie Structurale, McGill University, Montréal, Québec, Canada

Correspondence to:

Guy A. Rouleau,

Director, Montréal Neurological Institute and hospital,

Address: 3801, University Street, Office 636

Montréal, Québec H3A 2B4

Phone: +1-514-398-2690,

Fax. +1-514 398-8248

Email: guy.rouleau@mcgill.ca

Ziv Gan-Or

Department of Neurology and Neurosurgery

McGill University

1033 Pine Avenue, West,

Ludmer Pavilion, room 312

Montreal, QC, H3A 1A1,

Phone: +1-514-398-5845

Fax. +1-514 398-8248

Email: ziv.gan-or@mcgill.ca

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Abstract

Background

Although the typical inheritance of spastic paraplegia 7 is recessive, several reports have suggested that *SPG7* variants may also cause autosomal dominant hereditary spastic paraplegia (HSP).

Objectives

We aimed to conduct an exome-wide genetic analysis on a large Canadian cohort of HSP patients and controls to examine the association of SPG7 and HSP.

Methods

We analyzed 585 HSP patients from 372 families and 1175 controls, including 580 unrelated individuals. Whole-exome sequencing was performed on 400 HSP patients (291 index cases) and all 1175 controls.

Results

The frequency of heterozygous pathogenic/likely pathogenic SPG7 variants (4.8%) among unrelated HSP patients was higher than among unrelated controls (1.7%; OR 2.88, 95% CI 1.24–6.66, P = 0.009). The heterozygous SPG7 p.(Ala510Val) variant was found in 3.7% of index patients versus 0.85% in unrelated controls (OR 4.42, 95% CI 1.49–13.07, P = 0.005). Similar results were obtained after including only genetically-undiagnosed patients. We identified four heterozygous *SPG7* variant carriers with an additional pathogenic variant in known HSP genes, compared to zero in controls (OR 19.58, 95% CI 1.05–365.13, P = 0.0031), indicating potential digenic inheritance. We further identified four families with heterozygous variants in *SPG7* and *SPG7*-interacting genes (*CACNA1A*, *AFG3L2*, and *MORC2*). Of these, there is especially compelling

evidence for epistasis between *SPG7* and *AFG3L2*. The p.(Ile705Thr) variant in *AFG3L2* is located at the interface between hexamer subunits, in a hotspot of mutations associated with spinocerebellar ataxia type 28 that affect its proteolytic function.

Conclusions

Our results provide evidence for complex inheritance in *SPG7*-associated HSP, which may include recessive and possibly dominant and digenic/epistasis forms of inheritance.

Key Words: spastic paraplegia; HSP; SPG7; oligogenic inheritance

1 Introduction

Hereditary spastic paraplegia (HSP) is a group of rare neurodegenerative diseases considered to be inherited in a classical monogenic Mendelian manner. More than 80 loci/genes have been implicated in HSP, and some of these genes are also involved in diseases whose typical features differ from HSP.[1] HSP patients may present with a wide spectrum of symptoms, from very subtle lower limb spasticity to severe neurological and non-neurological manifestations. These symptoms often overlap with other disorders, which may lead to incorrect diagnoses.[2, 3]

Spastic paraplegia 7 (SPG7) is the first-identified autosomal recessive (AR) type of HSP, accounting for 5%–12% of AR-HSP.[4] Pathogenic variants in *SPG7* may also lead to spastic and/or cerebellar ataxia, peripheral neuropathy with no other neurological symptoms, primary progressive multiple sclerosis, amyotrophic lateral sclerosis, primary lateral sclerosis, parkinsonism, limb dystonia, and isolated dominant optic atrophy.[5-13] Although the inheritance of *SPG7* is considered to be AR, several reports suggested that *SPG7* variants may also cause autosomal dominant (AD) HSP.[5, 14-16] None of these studies examined the possibility of digenic inheritance (carrying two mutations in different HSP genes), therefore the role of *SPG7* in AD-HSP remains controversial.

SPG7 encodes the paraplegin/SPG7 protein, localized at the inner mitochondrial membrane. *SPG7* is comprised of 17 exons with several mutational hotspots.[17] The p.(Ala510Val) variant is a known hotspot in *SPG7*, which despite relatively high allele frequency (AF) in public databases (0.0027 in gnomAD controls) is considered pathogenic,[5, 16, 18] likely with incomplete penetrance or variable expressivity. The high frequency of the p.(Ala510Val) variant, the wide clinical spectrum, and the possibility of

dominant and recessive inheritance make diagnosis and genetic counseling in SPG7 challenging.

Herein, we performed a comprehensive genetic analysis in a large cohort of HSP patients. We examined whether heterozygous *SPG7* variants are overrepresented in HSP patients, and whether digenic inheritance of *SPG7* variants together with other HSP-related gene variants may occur in HSP.

2 Methods

2.1 Population

A total of 585 HSP patients from 372 families and 1175 control individuals (Tables S1 and S2, respectively) have been recruited across Canada, through the CanHSP consortium as previously reported,[19] and were analyzed for the presence of *SPG7* variants. Initially, 379 HSP patients were analyzed using different sequencing panels of known HSP genes, some included *SPG7* and some did not. Of those, 194 were not genetically diagnosed, and went through whole-exome sequencing (WES). An additional 206 HSP patients and 1175 individuals who served as a control group were sequenced directly using WES. These 1175 controls were collected at the laboratory of Dr. Guy Rouleau, and included healthy individuals and individuals with non-movement disorders that do not have any known overlap with HSP (details of these controls are in Table S2). Of the 1175 controls, 580 were unrelated individuals. Of all the unrelated control individuals (n = 580) and HSP index cases (n = 372, one from each family), 70% and

88.4% were Europeans, respectively, according to the HapMap Project data.[20] Principal component analysis (PCA, Fig. S1) shows overlapping main principal components. Since we study very rare variants in cases and controls, ethnicity is likely to have no or very minor effect. The diagnosis of HSP was based on previously published criteria.[21] Standardized clinical assessments were applied and included demographic characteristics, family history, pedigree, developmental history, and clinical symptoms. For a subset of patients, brain and spinal cord magnetic resonance imaging (MRI) were performed and the Spastic Paraplegia Rating Scale (SPRS) score was assessed.[22] All participants signed an informed consent form prior to enrollment, and the institutional review boards approved the study protocols.

2.2 Genetic and Data Analysis

DNA was extracted from peripheral blood using a standard salting-out procedure. In the HSP group, 379 patients were initially screened using panel sequencing of HSP-associated genes. In total, 1575 samples including 400 HSP and 1175 control samples went through WES. For exome target enrichment, SureSelect Human All Exon V4, V5, Nextera Rapid Capture Exomes, and TruSeq Exome (Illumina) kits were used, and the sequencing was performed on Illumina HiSeq 2000/2500 (San Diego, CA). The sequence reads were aligned to the human reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner.[23] Variant calling and annotation were done using Genome Analysis Toolkit and ANNOVAR, respectively.[24, 25]

Missense and truncating variants with minor allele frequency less than 0.01 in gnomAD were included in the analysis.[26] These variants were classified according to the

American College of Medical Genetics and Genomics guidelines using VarSome.[27] *SPG7* variants classified as “Variants of Unknown Significance”, “Likely Benign”, and “Benign” were excluded, and only “Pathogenic” and “Likely Pathogenic” variants were included. Intronic splicing variants at ≥±3 position with uncertain significance higher were also excluded. Variant calls with less than 30× depth of coverage, a genotype quality of less than 97, and less than 25% genotyping frequency were excluded from the analysis. All the detected variants were visually inspected with the Integrated Genomics Viewer and suspicious variants were validated using Sanger Sequencing. To check for relatedness in the control group and in HSP families with complex inheritance of *SPG7*, we used somalier.[28] To examine whether carriers of *SPG7* variants may carry other variants in other HSP-associated genes, or in genes linked to similar neurogenetic disorders, we screened for variants in 787 genes (Table S3)[29] in HSP patients who carried at least one *SPG7* allele.

Gene Ontology (GO) enrichment analysis was carried out using g:Profiler,[30] with Benjamini−Hochberg adjusted *P* values for statistical significance set at <0.05. For protein–protein interaction/network analysis, STRING[31] and GeneMANIA[32] were used. In order to predict the presence of important domains and sites of the corresponding protein, as well as multiple protein sequence alignment, we applied InterPro[33] and Clustal Omega tools, respectively.[34] A three-dimensional atomic model of tubulin cofactor E (TBCE) (a.a. 97–443) was built using the automated server I-TASSER.[35] The model was derived from the coordinates of different leucine-rich repeat (LRR) domains sharing 12%–20% identity. A second model of the TBCE LRR domain (a.a. 97–347) was also built with SWISS-MODEL[36] with the structure of the plant receptor BRI1 (pdb 3rj0, 33% sequence identity

for this segment). The atomic coordinates of the TBC CAP-Gly domain (pdb 4b6m), TBCE Ubl domain (pdb 4icu), and human AFG3L2 (pdb 6nyy) were downloaded from the Protein Data Bank. The steric clashes induced by each mutation were evaluated using the "mutagenesis" toolbox in PyMol v. 2.3.5.

To study genotype–phenotype correlations in SPG7, we removed carriers of other pathogenic/likely pathogenic variants in HSP-related genes to avoid bias by other, non-*SPG7* variants. We compared the following groups of patients: (1) carriers of homozygous variants; (2) carriers of compound heterozygous variants; and (3) carriers of heterozygous variants. Since it was previously suggested that HSP patients with the p.(Ala510Val) variant have a milder phenotype,[37] we also compared carriers of this variant to other SPG7 patients. In addition, due to the report of differences in clinical presentations between SPG7 patients with loss-of-function (LoF) and missense variants, patients were classified and compared based on the variant type including patients with: (1) one missense; (2) one LoF; (3) one missense and one LoF; (4) two missense; and (5) two LoF variants.

2.3 Statistical Analysis

For binary variables, chi-square and Fisher's exact test were used, and for continuous variables Mann−Whittney U and Kruskal−Wallis tests were used, as required. SPSS was used to perform all statistical analyses. For the genotype–phenotype analysis of symptoms, Bonferroni correction for multiple comparisons was applied and corrected *P* value threshold was set to <0.0005.

3 Results

3.1 Bi-Allelic and Monoallelic *SPG7* Variant Carriers

Of 585 HSP patients, potentially pathogenic rare *SPG7* variants were identified through either panel sequencing or WES in 38 patients (6.5%) with homozygous or compound heterozygous variants and in 21 patients (3.6%) with heterozygous variants. In order to further compare frequencies of *SPG7* variants between patients and controls, we only included samples that went through WES, since controls did not go through panel sequencing, and since different sequencing panels were used, some of which did not include SPG7. In index (unrelated) cases of HSP who underwent WES (n = 291), 48 pathogenic/likely pathogenic *SPG7* alleles were detected compared to 10 in unrelated controls (n = 580, OR 11.25, 95% CI 5.60–22.62, P < 0.0001). After excluding biallelic *SPG7* mutation carriers, 13 (of n = 270, 4.81%) heterozygous pathogenic/likely pathogenic *SPG7* variant carriers were identified versus 10 (1.72%) carriers which were identified among 580 unrelated controls (OR 2.88, 95% CI 1.24–6.66, *P* = 0.009). The heterozygous *SPG7* p.(Ala510Val) variant was found in 10 (3.70%) of the index cases versus 5 (0.85%) in unrelated controls (OR 4.42, 95% CI 1.49–13.07, *P* = 0.005). We further examined the role of heterozygous pathogenic/likely pathogenic *SPG7* variants only in genetically undiagnosed HSP patients. Of 148 index cases with genetically undiagnosed HSP, 10 (6.76%) carried an *SPG7* variant compared to 10 (1.72%) among the 580 unrelated controls (OR 4.13, 95% CI 1.69–10.12, P = 0.0026). Among the 148 genetically undiagnosed HSP patients, the *SPG7* p.(Ala510Val) variant was found in 8

(5.41%) compared to 5 (0.85%) in unrelated controls (OR 6.57, 95% CI 2.12–20.39, P = 0.0013). Table S4 and Figure 1 details these variants in all cohorts.

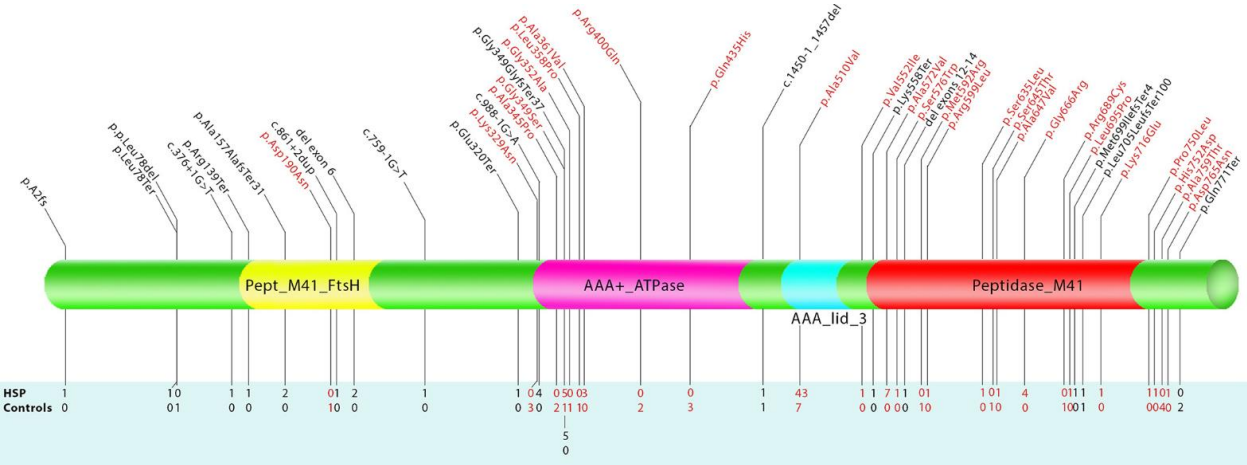

FIG. 1

Schematic representation of the locations of *SPG7* variants in hereditary spastic paraplegia (HSP) patients and controls. InterPro predicted four domains for spastic paraplegia 7 (SPG7) including Pept_M41, AAA + _ATPase, AAA_lid_3, and Peptidase_M41 domains. Missense variants are indicated in red while loss of function variants are in black. The numbers below represent the number of individuals carrying the specific variant.

3.2 Evidence of Potential Digenic Inheritance and Modifier Effects in *SPG7*-Associated HSP

We further hypothesized that the overrepresentation of heterozygous *SPG7* variants in HSP patients versus controls could be due to digenic inheritance, namely that patients with heterozygous *SPG7* variants may also carry variants in other HSP or similar neurogenetic disorders-associated genes (Table S3). We found four index cases with

heterozygous *SPG7* variant who also carried pathogenic variants in other genes that are associated with spastic paraplegia/ataxia (Table 1) compared to zero in controls (OR 19.58, 95% CI 1.05–365.13, *P* = 0.0031; Fisher's exact test after Haldane−Anscombe correction). Of note, the penetrance of HSP-related mutations is not necessarily complete,[39] therefore there could be controls with such mutations. One of these four families (01-013) had two affected members who carried a pathogenic variant in *BSCL2*. One of the affected members of this family carried an *SPG7* p.(Ala510Val) variant while the other one was a non-carrier. In addition to the core symptoms, the *SPG7* variant carrier also presented with swallowing difficulties, upper extremity weakness, hyperreflexia, and lower motor neuron features. One patient with heterozygous *SPG7* variant also carried two variants in *TBCE* (Table 1), which is known to cause encephalopathy with spasticity. One of the two *TBCE* variants was annotated as pathogenic, while the other was annotated as likely benign. This patient presented with clinical features that fit both *SPG7* and *TBCE* (Table 1). In addition, this patient presented with nephropathy and focal segmental glomerulosclerosis, which have not been previously reported in SPG7 and are a very rare clinical finding in HSP.[40] We could not find a pathogenic variant in a gene related to nephropathy in this patient. Two other patients carried *SPAST* mutations (Table 1). The average age at onset (AAO) of these four potentially digenic patients was 2.7 ± 2.1 years compared to 28.0 ± 17.9 years in all other patients with *SPG7* variants (*P* = 0.060, Mann−Whitney U test). Upper extremity weakness (2/2 vs. 3/38, *P* = 0.013) and hyperreflexia (3/3 vs. 11/39, P = 0.032) were more common among the potentially digenic patients with available data. None of these differences was statistically significant after correction for multiple comparisons.

TABLE 1. Genetic and clinical characteristics of hereditary spastic paraplegia (HSP) patients who carried at least one *SPG7* allele along with pathogenic non-spastic paraplegia 7 (*SPG7*) variant(s) that could explain the disease

CanHSP	Symptoms	OMIM	Identified Gene Variant (Inheritance mode)	Novelty	VarSome	CADD	MutationTaster	Identified *SPG7* variants (Inheritance mode)	VarSome / Pathogenicity of *SPG7* variant in previous studies
FSP 01-013	LEW, LES, LEH, Extensor Plantar Responses, Abnormal Bladder Function, Ankle Clonus, swallowing difficulties, upper extremity weakness, upper extremity hyperreflexia, amyotophy or lower motor neuron features, Abnormal EMG results AAO: 5	LEW, LES, LEH, Hyperreflexia, Extensor plantar responses, Pes cavus, Decreased vibratory sense, Distal limb muscle weakness and atrophy, AAO: 8-40	***BSCL2*** NM_032667 c.269C>T;(p.[Ser90Leu]) (Het)	Reported[38]	Pathogenic	34	0.5876 (Disease Causing)	c.1529C>T;(p.[Ala510Val]) (Het)	Pathogenic / [5,18]
FSP 01-171	LES, LEH, Abnormal Bladder Function, Ankle Clonus,	LEW, LES, LEH, Extensor Plantar Responses, Pyramidal	***SPAST*** NM_014946 c.1212_1216del; (p.[Asn405LysfsTer36])	Novel	Pathogenic	-	-	c.1529C>T; (p.[Ala510Val]) (Het)	Pathogenic / [5,18]

	upper extremity hyperreflexia, Mild vibratory loss AAO: 2	signs, Cognitive decline, Decreased vibratory sense, Deficits in language expression, AAO: variable	(Het)						
FSP 04-012	LEW, LES, LEH, Extensor Plantar Responses, Ankle Clonus, Speech Delay or Abnormality, Learning Disability, progressive cognitive deficits, deafness, dysarthria, upper extremity weakness, upper extremity hyperreflexia, nephropathy, Focal segmental glomerulosclerosis SPRS: 37 AAO: 1	Distal amyotrophy, Encephalopathy, Delayed psychomotor development, Motor regression, Spinal muscular atrophy, Intellectual disability, Ataxia, Spastic tetraplegia, Dysarthria, Absence of speech, Cerebellar atrophy, Thin corpus callosum, Axonal peripheral neuropathy, AAO: Infantile	***TBCE*** NM_003193 c.661G>C; (p.[Val221Leu]) & c.1537C>T; (p.[Gln513Ter]) (Comp Het)	Novel & Novel	Likely Benign & Pathogenic	15.44 & 41	0.5876 (Disease Causing) & 0.81 (Disease Causing)	c.1796G>T; (p.[Arg599Leu]) & c.184-3C>T (Comp Het)	Likely Pathogenic / - & Uncertain Significance /

Abbreviations: OMIM, Online Mendelian Inheritance in Man; CADD, Combined Annotation-Dependent Depletion; LEW, lower extremity weakness; LES, lower extremity spasticity; LEH, lower extremity hyperreflexia; Het, heterozygous; Comp

Het, compound heterozygous; AAO, age at onset (years); EMG, electromyography; NA, not available; SPRS, Spastic Paraplegia Rating Scale.

To examine whether *SPAST*, *BSCL2*, and *TBCE* (the genes in which we found additional mutations in patients who also carry *SPG7* heterozygous mutation, Table 1) are involved in specific cellular pathways in which SPG7 is also involved, we performed pathway enrichment analysis. We found enrichment mainly in axonal transport and cellular organization (adjusted $P < 0.05$, Table S5), with *SPAST* and *SPG7* having the highest enrichment. Both proteins play a role in axonal transport and involved in biosynthesis, assembly, and arrangement of macromolecules and cellular membrane and other components (Table S5). Spastin (SPG4) and paraplegin (SPG7) contain the same ATPase domain where they participate in diverse cellular processes (IPR003959, IPR003593).

To identify additional patients who potentially have HSP due to digenic inheritance involving *SPG7* we examined very rare variants (AF < 0.001) in genes that produce proteins that interact with *SPG7* (Table S6). We found very rare heterozygous variants with uncertain significance in three genes (*CACNA1A*, *AFG3L2*, and *MORC2*) in heterozygous carriers of *SPG7* (Table 2). Among them, *AFG3L2* had the closest interaction with *SPG7* and both share similar features, including protein structure and domains, interacting proteins, and biological functions and pathways (Table S7). Except for one homozygous *AFG3L2* variant, c.122G > A;(p.[Arg41Gln]) with gnomAD AF 0.0000048, which was identified in one control (who did not carry an *SPG7* variant) out of 1175, no rare variants were detected in *CACNA1A*, *AFG3L2*, and *MORC2* in our controls. Yet, to confirm their potential pathogenicity in digenic HSP, additional genetic and functional studies are needed. Of note, we have also identified three controls with

rare variants in the genes *ATP13A2* (p.[Val803Ile]), *FARS2* (p.[Ala302Ser]), and *MTUS2* (p.[Arg1091Leu]), which may also interact with *SPG7*.

TABLE 2. Variants in *SPG7* interacting genes which were identified in hereditary spastic paraplegia (HSP) patients with heterozygous *SPG7* variants

Patient	Gene (known phenotype)	Variant	gnomAD (non-neuro) AF	CADD	MutationTaster	SIFT	GERP	SPG7 variant	Evidence of interaction with SPG7
FSP-04-053	*AFG3L2* (Spinocerebellar ataxia 28 # 610246)	NM_006796 c.2114T>C;(p.[Ile705Thr]) *	0	29.1	0.81 (Disease causing)	0.006 (Damaging)	5.6	c.376+1G>T	[41-43]
FSP-01-190	*CACNA1A* (Spinocerebellar ataxia 6 # 183086)	NM_001127221 c.4981C>T;(p.[Arg1661Cys])	0	25.3	0.81 (Disease causing)	0 (Damaging)	4.9	p.(Ala510Val)	[44]
FSP-04-045	*MORC2* (Charcot-Marie-Tooth disease, axonal, type 2Z # 616688)	NM_014941 c.2716C>T;(p.[Arg906Cys])	9.61e-6	23.7	0.5876 (Disease causing)	0.19 (Tolerated)	4.3	p.(Lys716Glu)	GeneMANIA

[a] The variant was reported from one hereditary ataxia patient[45].

Abbreviations: AF, allele frequency; CADD, Combined Annotation-Dependent Depletion; SIFT, Sorting Intolerant From Tolerant; GERP, Genomic Evolutionary Rate Profiling; SPG7, spastic paraplegia 7; CMT, Charcot–Marie–Tooth.

3.3 Family History in Carriers of Heterozygous and Potential Digenic *SPG7* Variants

Families with heterozygous *SPG7* variants, with or without variants in other genes, showed different modes of inheritance, further supporting potential complex and/or non-Mendelian inheritance associated with *SPG7* (Fig. S2). For example, family 03-019 had a dominant model of inheritance, and three individuals from three different generations carried the *SPG7* p.(Ala510Val) variant, and no other variants that can explain this inheritance. In families 01-090 and 02-048, the index cases also carried the same variant, and in these families too the inheritance seems to be dominant. In family 01-190, the index case carried the *SPG7* p.(Ala510Val) variant and the *CACNA1A* p.(Arg1661Cys), and while there are patients in each of the three generations documented in this pedigree, the inheritance could only be dominant with partial penetrance, as the mother of the index case (indicated with an arrow) was not reported to have the disease at the time the data were collected. Other families, such as 01-009, 01-013, and 01-052, may suggest incomplete inheritance, digenic inheritance, or other forms of complex inheritance (Fig. S2).

3.4 Structural Analysis of *TBCE* and *AFG3L2* Variants

Among the non-HSP genes that may contribute to oligogenic inheritance or epistasis, *AFG3L2* and *TBCE* were the strongest candidates. To further examine whether the two *TBCE* variants identified in family 04-012 (Table 1) may be pathogenic, we performed structural analysis of their potential effects on TBCE structure and function. The human tubulin cofactor E (TBCE) consists of an N-terminal CAP-Gly domain, followed by an LRR domain and a C-terminal ubiquitin-like (Ubl) domain (Fig. 2A). The

structures of TBCE alone and in complex with α-tubulin and tubulin folding cofactor B (TBCB) were determined at low resolution by electron microscopy.[46] The complex structure shows that α-tubulin binds to the CAP-Gly domain as well as the concave surface of the LRR domain. This interaction enables the TBCE−TBCB complex to dissociate the α/β tubulin heterodimers into monomers that can be degraded by the ubiquitin−proteasome system.[47] To investigate the impact of the potential HSP-related variant p.(Val221Leu) on TBCE's structure and function, we performed in silico mutagenesis of the residue in two homology models of TBCE. Val221 is located on the "exterior" side of the LRR domain, opposite to the side of interaction with tubulin (Fig. 2A). In the homology model generated with I-TASSER, the side chain of Val221 points towards the solvent. The variant Val to Leu results in a clash with a helix in an adjacent repeat (Fig. 2B). In the homology model generated with SWISS-MODEL using the homologous LRR domain of the plant steroid hormone receptor BRI1 (pdb 3rj0), Val221 is located in the hydrophobic core, and the p.(Val221Leu) variant also leads to steric clashes (Fig. 2C). In both models, our prediction is that this variant destabilizes the domain and might inactivate it. The mutation p.(Gln513Ter) would result in a 15 amino acid deletion in the Ubl domain of TBCE (Fig. 2A) The crystal structure of the Ubl (pdb 4icu) shows that this segment comprises a helix and the C-terminal β strand, which is typically involved in protein–protein interactions.[48] Deletion of this segment would therefore completely unfold the Ubl and disrupt its function. The Ubl domain might be involved in shuttling tubulin to the proteasome, given that the proteasome subunit Rpn10 binds to ubiquitin and Ubl domains.[49, 50] However, the Ubl domain of TBCE has only 21% sequence identity with ubiquitin, and comparison of the TBCE Ubl with the complex of Rpn10 and the Ubl of

UBQLN2 reveals that most residues interacting with Rpn10 are not conserved between UBQLN2 and TBCE. Therefore, the TBCE Ubl is unlikely to bind to the proteasome and its function remains unknown.

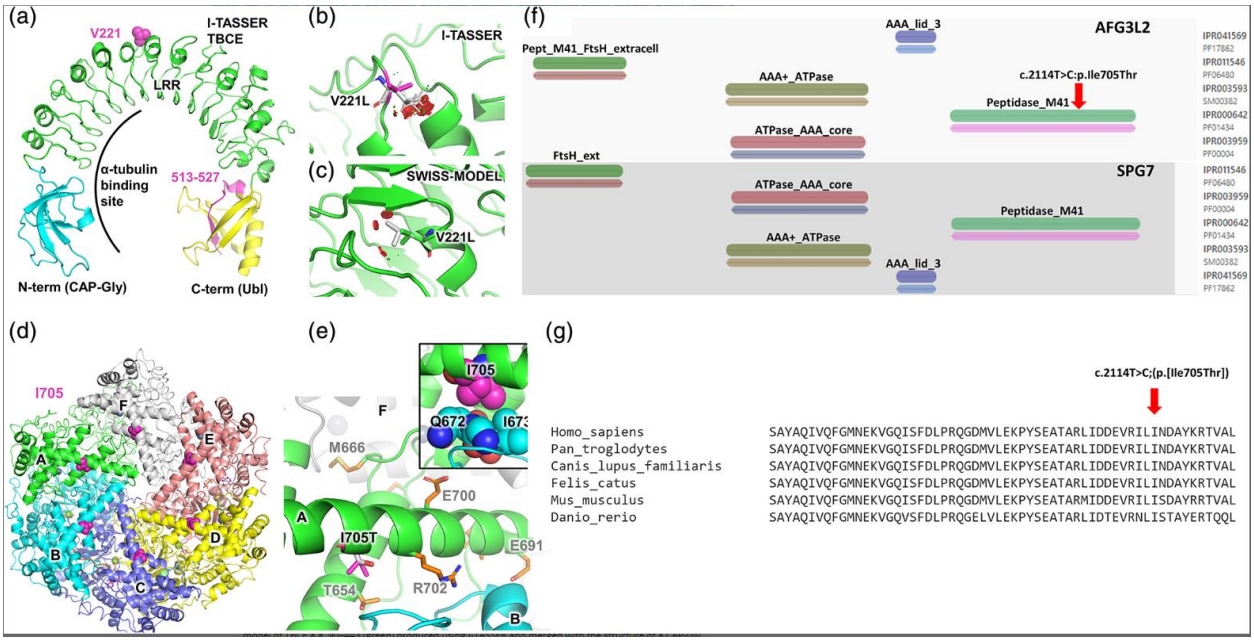

FIG. 2

Protein structural analysis of the identified variants in tubulin cofactor E (TBCE) and AFG3L2. (A) Homology model of TBCE a.a. 90–443 (green) produced using I-TASSER and merged with the structure of a CAP-Gly domain (a.a. 1–90, cyan, pdb 4b6m) and crystal structure of the C-terminal ubiquitin-like (Ubl) domain (a.a. 444–527, yellow, pdb 4icu). Variant sites are in magenta. Val221 is in the leucine-rich repeat (LRR) domain. The p.Gln513Ter variant removes a.a. 513–527, which form part of a helix and the C-terminal β strand. The α-tubulin binding site derived from a low-resolution electron microscopy (EM) structure is shown. (B) Close-up view of the p.Val221Leu variant site in the I-TASSER model. The mutated residue is shown in white. The variant would create clashes (red disks) with a helix in an adjacent repeat. (C) Close-up view of the

p.Val221Leu variant site in the SWISS-MODEL homology model. Here the side chain of Val221 points in the hydrophobic core, and the variant results in clashes as well. Cartoon images were produced using PyMOL v.2.3.5. (D) Cryo-electron microscopy (cryo-EM) structure of human AFG3L2 (pdb 6nyy). The protein forms a homo-hexamer with the six chains labeled A−F. Ile705 is shown in magenta. (E) Close-up view of the p.Ile705Thr variant site. The side chain of Ile705 in chain A makes VdW contacts with a loop in chain B (small inset, top right). The variant (white) introduces no clash, but makes the interface less hydrophobic. SCA28 variant sites are shown in orange. Cartoon images produced using PyMOL v.2.3.5. (F) Domain prediction by InterPro revealed that SPG7 and AFG3L2 share similar protein domains. The variant in AFG3L2 occurred in the peptidase M41 domain (IPR000642) which belongs to metallopeptidase family. (G) Sequence alignment of AFG3L2 orthologs, showing conservation of Ile705. The AFG3L2 variant occurred in an amino acid that is highly conserved among species.

AFG3L2 is a subunit of the m-AAA protease complex, which cleaves proteins in the mitochondrial inner membrane.[51] It consists of an N-terminal segment anchored to the membrane, followed by an ATPase and protease domains that assemble into a hexamer. The structure of the soluble domains of human AFG3L2 bound to a substrate was determined by cryo-electron microscopy (cryo-EM) at high resolution and revealed how its protease and ATPase domains coordinate to pull in substrates for proteolysis.[52] The mutation p.(Ile705Thr) locates to a helix in the central protrusion of the protease domain at the interface of the protease in another subunit (Fig. 2D). The side chain of

Ile705 forms hydrophobic interactions with a loop formed by Gln672 and Ile673 (Fig. 2E). The mutation p.(Ile705Thr) results in no steric clash, but would make the interface less hydrophobic. Intriguingly, Ile705 is located in a “hotspot” of SCA28-associated mutations such as p.(Thr654Ile), p.(Met666Arg/Val/Thr), p.(Pro688Thr), p.(Tyr689His/Asn), p.(Glu691Lys), p.(Glu700Lys), and p.(Arg702Gln) (Fig. 2E). All of these mutations strongly reduce the ATPase and degradation rates of AFG3L2, and the mutations p.(Met666Arg), p.(Pro688Thr), and p.(Glu691Lys) prevent formation of hexamers.[52] Thus, p.(Ile705Thr) may also affect the stability of the AFG3L2 hexamer and may compromise the functional coupling between the protease subunits.

3.5 Genotype–Phenotype Correlations Among *SPG7* Mutation Carriers

Clinical data were available for 12 heterozygous carriers, nine homozygous carriers (seven of whom are homozygous carriers of the p.(Ala510Val) variant), and 22 compound heterozygous carriers. Table 3 details the clinical data for each of these groups, each time comparing one group to the other two. After correction for multiple comparisons, there were no statistically significant differences between the groups. However, some differences between heterozygous carriers and biallelic variant carriers are notable. While upper extremity ataxia (37.9%) and intent tremor (30%) were relatively common in biallelic carriers of *SPG7* variants, none of the heterozygous carriers showed these symptoms. Two heterozygous carriers presented with motor developmental delay, which was not found in any biallelic patients. Patients with heterozygous *SPG7* variants had younger AAO compared to biallelic patients (16.5 vs. 33.8 years, P = 0.021).

Cerebellar atrophy was the most common imaging finding among biallelic patients (22.7%).

TABLE 3. Genotype–phenotype correlation analysis in hereditary spastic paraplegia (HSP) patients according to SPG7 variants status

Variable	Heterozygous (n=17)	Homozygous (n=12)	Compound Heterozygous (n=26)	*P value*		
				Het vs Homo	Het vs Comp Het	Homo vs Comp Het
Age at onset ± SD (years)	16.5 ± 19.4 (n=12)	37.1 ± 17.4 (n=9)	30.5 ± 14.5 (n=22)	0.009	0.074	0.174
Male/female ratio	0.83 (n=11)	0.37 (n=11)	4.25 (n=21)	0.659	0.056	0.006
Spastic Paraplegia Rating Scale (SPRS) score	25.8 ± 11.4 (n=5)	14.50 ± 6.30 (n=8)	17.3 ± 6.9 (n=14)	0.093	0.156	0.482
Lower extremity weakness	8/12	8/9	15/21	0.338	1.00	0.393
Lower extremity spasticity	11/12	9/9	20/21	1.00	1.00	1.00
Lower extremity hyperreflexia	10/12	9/9	20/21	0.486	0.538	1.00
Extensor plantar responses	10/11	8/9	18/21	1.00	1.00	1.00
Abnormal bladder function	8/11	3/9	8/21	0.175	0.135	1.00
Ankle clonus	5/10	7/9	15/20	0.350	0.231	1.00
Motor delay	2/8	0/9	0/21	0.206	0.069	-
Learning disability	0/7	0/9	1/21	-	1.00	1.00
Progressive cognitive deficits	1/9	2/9	0/21	1.00	0.30	0.083
Retinopathy or optic atrophy	0/9	0/9	1/20	-	1.00	1.00
Ocular movement abnormalities	0/9	1/9	7/21	1.00	0.071	0.374
Deafness	0/9	1/9	0/20	1.00	-	0.310
Swallowing difficulties	1/9	0/9	2/20	1.00	1.00	1.00
Dysarthria	1/9	3/9	9/20	0.576	0.107	0.694
Upper extremity weakness	0/9	1/9	2/20	1.00	1.00	1.00
Upper extremity hyperreflexia	3/9	3/9	5/21	1.00	0.666	0.666
Amyotrophy or lower motor neuron features	1/9	1/9	3/19	1.00	1.00	1.00
Sensory abnormalities	2/9	3/9	6/21	1.00	1.00	1.00
Peripheral neuropathy	3/8	1/8	4/19	0.569	0.633	1.00

Pes cavus	4/9	3/9	4/20	1.00	0.209	0.642
Ataxic gait	1/9	4/9	9/20	0.294	0.107	1.00
Upper extremity ataxia	0/9	3/9	8/20	0.206	0.033	1.00
Upper extremity intent tremor	0/9	2/9	7/21	0.471	0.071	0.681
Lower extremity ataxia	1/9	3/9	10/20	0.576	0.096	0.454
Lower extremity intent tremor	1/9	3/9	7/20	0.576	0.371	1.00
Seizures	0/9	0/9	1/20	-	1.00	1.00
Skeletal abnormalities	1/9	0/9	1/20	1.00	0.310	1.00
Myoclonus	1/9	0/9	0/19	1.00	0.321	1.00
Abnormal brain MRI	1/6	3/5	4/17	0.242	1.00	0.274
Abnormal spine MRI	1/6	0/6	0/15	1.00	0.286	-
Rare symptoms	Flexar plantar response, Scoliosis, Polyneuropathy, Dystonia in hands, saccadic on pursuit, Bilateral cataract	Leukoencephalopathy, very mild cerebellar in arms and legs, Jaw Jerk	Leukoencephalopathy, Dopa-responsive parkinsonism, Ankle weakness, dysphagia, dysmetria, Jaw Jerk, bilateral carpel tunel, ptosis, ophthalmoplegia, Dystonia in hands			

The corrected P value threshold was $P < 0.0005$. None of the clinical features significance passes Bonferroni correction.

Abbreviations: SD, standard deviation; SPRS, Spastic Paraplegia Rating Scale; Het, heterozygous; Homo, homozygous; Comp Het, compound heterozygous; MRI, magnetic resonance imaging.

We further examined whether there are differences between subgroups of patients, based on the type of variants that they carried (missense, LoF) and the presence of the most common variant, p.(Ala510Val), which was carried by 34 (53.9%) patients with at least one allele. No statistically significant differences were identified after correction for multiple comparisons (Table 3, Table S8), likely due to the small number of patients in each of these groups.

4 Discussion

The current study summarizes genetic and clinical data on SPG7 from a large Canadian cohort of 585 patients, of which 6.5% carried biallelic *SPG7* variants. Our findings show that the number of pathogenic/likely pathogenic *SPG7* alleles in HSP patients is higher than in controls, even when considering only heterozygous carriers in index HSP patients versus unrelated controls, suggesting potential dominant or digenic inheritance in some cases. We also found that some of the heterozygous carriers of *SPG7* pathogenic variants with HSP carried other potentially pathogenic variants in other genes, a phenomenon which was not observed in controls. These findings, which require replication, may suggest digenic inheritance in HSP associated with *SPG7*. Of note, we cannot rule out that in some of the heterozygous carriers of *SPG7* variants an additional *SPG7* variant exists that was not detected through WES.

Our results therefore suggest that non-Mendelian inheritance may have a role in SPG7-associated HSP, and it should be considered in HSP in general. In classic Mendelian inheritance, a single gene is associated with a single trait. As shown here, it is possible that more than one gene is involved in SPG7-associated HSP. The simplest form

of non-Mendelian inheritance is digenic inheritance, in which a combination of two genes may lead to a disease.[53] The patients with mutations in both *SPG7* and other genes as detailed below could be examples of such inheritance. More complex forms of non-Mendelian inheritance may also exist,[54] and their potential role in HSP should be further investigated.

The four patients with heterozygous *SPG7* variants who also carried other variants in genes related to HSP had younger AAO (2.7 vs. 28 years) and higher average SPRS score (37 vs. 18.1), indicating that their disease may be more severe than those who did not carry variants in two genes. We further examined the possibility of genetic interaction/modification by performing biological pathway enrichment analysis. *SPAST* and *SPG7*, whose variants co-occurred in three HSP patients, share a similar ATPase domain and closely interact in multiple pathways. In the control cohort, none of the participants carried pathogenic variants in the genes which were identified in the HSP patients (*SPAST*, *BSCL2*, and *TBCE*). Overall, these findings may suggest either digenic inheritance, or epistasis between heterozygous *SPG7* variants and other HSP-related genes, as previously reported in SPG4.[55-57] Digenic inheritance has been suggested in spinocerebellar ataxia and Charcot−Marie−Tooth (CMT), diseases initially considered as a simple Mendelian monogenic disorder.[58, 59] Recently it was demonstrated that the *EXOC4* gene may be involved in complex inheritance in axonopathies, including HSP and CMT.[60]

By combining interaction and genetic analyses, we identified heterozygous carriers of *SPG7* who also carried variants in genes potentially interacting with *SPG7*, including *AFG3L2*, *CACNA1A*, and *MORC2*. It is possible that the co-occurrence of the

heterozygous variants of these genes with *SPG7* heterozygous variants may lead to HSP. These results require replication in additional cohorts and additional functional evidence. The pathway enrichment, domain prediction (Fig. 2F), protein network, and protein conservational analysis (Fig. 2G) showed that AFG3L2 is the strongest potential candidate interacting with SPG7. This is further supported by functional studies, demonstrating the potential interaction of these two proteins within the mitochondria.[41, 61] SPG7 and AFG3L2 exert overlapping substrate specificities, hence the expression level of AFG3L2 and SPG7 might be important in cell-type specificity in disorder. Dominant *AFG3L2* mutations cause spinocerebellar ataxia type 28, whereas bi-allelic mutations may affect the interaction of SPG7 and AFG3L2 and cause spastic ataxia 5, a disease whose phenotype includes features of both SCA28 and SPG7. A recent study reported a patient with heterozygous variants in both genes with syndromic parkinsonism and optic atrophy.[42] SPG7 and AFG3L2 are components of mitochondrial m-AAA proteases and they can assemble hetero-oligomeric proteolytic complexes with SPG7.[41] Together with the current study, these data suggest that *SPG7-AFG3L2* digenic variants may be a cause of HSP and similar disorders, and that individuals with heterozygous *SPG7* variants with neurodegeneration should be specifically screened for *AFG3L2* variants.

We also report several rare clinical features of SPG7, including dopa-responsive parkinsonism, dysphagia, dysmetria, jaw jerk, ptosis, ophthalmoplegia, optic atrophy, and hands dystonia in biallelic *SPG7* patients. A few studies previously reported some of these symptoms in patients with *SPG7* mutations.[6, 62-65] Optic atrophy and dopa-responsive parkinsonism were also reported in a patient with concurrent *AFG3L2* and *SPG7* heterozygous variants.[42] Similar to other genes involved in HSP, SPG7 may be

involved in a continuum of spastic neurogenetic disorders, and *SPG7* variants should not be ruled out only because of the presence of rare clinical manifestations.

Our study has several limitations. Despite being one of the world's largest HSP cohorts, the total number of SPG7 patients is still relatively small, especially for genotype–phenotype studies. In addition, not all our HSP cohort went through WES, which prevented the participation of all patients in some of the analyses. One limitation of WES is that it cannot properly detect genetic variants such as large copy number variants (CNVs). While we did analyze the data with ExomeDepth,66 a computational tool for CNV detection using WES data, and did not identify CNVs, we cannot rule out that some of our supposedly heterozygous carriers of *SPG7* variants carry an additional undetected *SPG7* variant. For example, a recent study demonstrated that a deep intronic *SPG7* variant that led to inclusion of pseudoexon and early termination of the SPG7 protein could not be detected through WES and caused HSP.[67] An additional limitation of our study is the lack of age- and sex-matched controls. The male:female ratio in patients is 0.83 and in controls it is 1.08, both relatively close to 1 but with opposite direction. The average age at onset of patients was 22.1 years, and the age of controls was mostly unknown. However, all controls were >18 years old and most had children or grandchildren at recruitment, suggesting that they were likely older than 22 years on average. Yet this should still be considered as a limitation of the current study, as accurate data are not available.

To conclude, our results suggest that the inheritance of *SPG7* may be complex, and also include dominant or digenic inheritance. One of the most intriguing findings, which requires replication, is the potential digenic inheritance with *AFG3L2* variants. The relatively high allele frequency of some of the pathogenic *SPG7* variants in the general

population, the results of the genotype–phenotype correlation analysis, and a recent functional study[42] support this possibility. Future studies will benefit from whole-genome sequencing, which will allow for identifying CNVs, and deep intronic variants that can lead to aberrant splicing, as well as for comprehensive investigation of complex inheritance in SPG7 and other forms of HSP.

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Authors' Roles

Study concept and design: Z.G.O., G.A.R., M.A.E.;

Acquisition of data: I.H.S., A.D., G.Y., M.T., K.M.B., N.D., O.S., G.A.R.;

Analysis and interpretation of data: M.A.E., Z.G.O., E.Y., E.L., J.P.R., K.M., F.A., E.L., D.S., J.A.R., F.A., P.A.D., J.F.T.;

Writing of the first draft: M.A.E., Z.G.O., E.Y.;

Drafting of the manuscript: Z.G.O., M.A.E., J.F.T., K.M.B., M.T., O.S., G.Y., G.A.R.;

Critical revision of the manuscript for important intellectual content: G.A.R., Z.G.O., G.Y., M.T., K.M.B., N.D., P.A.D., O.S.;

Statistical analysis: D.S., F.A., J.P.R., I.H.S.;

Administrative, technical, or material support: E.L., F.A., J.A.R., P.A.D., J.F.T.;

Study supervision: G.A.R., Z.G.O.

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CHAPTER 6: GENERAL DISCUSSION

The major goal of this PhD project was to further expand the genetic spectrum of HSPs and to provide novel insights into its genetic causes. In our cohort, 330 potentially pathogenic variants across 79 genes were found, 164 of which were not reported in gnomAD. The variants were 197 missense, 37 nonsense, 28 splice site, 40 frameshift indels, 7 in-frame indels, 2 synonymous, 2 deep intronic, 1 start loss and 16 CNVs, some of which may not be detectable even using ES. Our findings revealed a new perspective that is beyond a simple Mendelian inheritance in HSPs. We also reported novel or extremely rare symptoms and clinical signs in HSP subtypes. For example, we described dopa-responsive parkinsonism, dysmetria, dysphagia, ptosis, jaw jerk, ophthalmoplegia, and hands dystonia in SPG7, deafness, ocular movement abnormality, upper extremity intention tremor, and seizure in SPG4, hammer toes in SPG77, ataxia in SPG52 and psychiatric symptoms and seizure in SPG78. Moreover, in SPG78 patients, we reported a patient with a frameshift *ATP13A2* mutation who presented with an HSP-predominant phenotype (Estiar *et al.*, 2020) while the previous patient who carried the same mutation, manifested a Parkinsonism-dominant phenotype (Eiberg *et al.*, 2012). This raises an important question of why the same mutation could be associated with various clinical presentations. It suggests that other factors contribute to the phenotype, contradicting a simple Mendelian inheritance model. Along the same lines, our findings from *SPG7* carriers challenge the concept of a classical monogenic inheritance pattern in HSPs (Estiar *et al.*, 2021). One of the findings was a potential digenism between SPG7 and its biological counterpart, AFG3L2. The pathogenic *SPG7* and *AFG3L2* variants were found in 10 additional patients from various motor neuron disorders cohorts versus none in

controls (unpublished data). SPG7 performs its main function by assembling with the homologous AFG3L2 and form a heterohexamer with AFG3L2 (Atorino *et al.*, 2003; Koppen *et al.*, 2007). Both SPG7 and AFG3L2 have the highest expression level in the cerebellum and cerebellar hemisphere compared to other regions of the brain (GTEx) and both encode highly similar proteins with the same conserved domains. Consistently, our findings from an additional 11 HSP patients with potentially pathogenic variants in two distinct genes associated with HSP or related phenotype further supported oligogenic causation or epistasis. Findings from other studies using burden analysis also highlighted an oligogenic model in historically Mendelian disorders (Bis-Brewer *et al.*, 2020). Besides complex inheritance that may challenge genetic counseling in HSP, multiple mode of inheritance was also seen in our study. We found that the number of heterozygous *SPG7* variants is significantly higher in HSP patients versus control individuals. Consistently, AD-SPG7 was seen in several families (Arnoldi *et al.*, 2008; Sánchez-Ferrero *et al.*, 2013). Two patients in our cohort had homozygous *ATL1* and *SPAST* variants that are known to be involved in AD-HSPs. Heterozygous *MARS* variants are known to be involved in CMT (OMIM # 616280). However, our two unrelated probands along with a patient from another study (Novarino *et al.*, 2014a) harbored biallelic *MARS* variants with HSP manifestation. Therefore, we suggest not excluding genetic tests on patients based solely on the trait of inheritance or clinical characteristics.

In AR-HSPs, the probability of undetected secondary alleles should be also taken into account, particularly in SPGs with both AR and AD patterns. More genes could be added to this list in the future. Therefore, we strongly suggest full screening and re-examination of the whole gene before excluding heterozygous carriers from further analysis or

concluding that a heterozygous variant is sufficient to cause the disease. The latter concept was further highlighted by the identification of a deep intronic splice variant in a carrier of missense *SPG7* allele (Verdura *et al.*, 2020). Similarly, a deep intronic variant *POLR3A*:c.1909+22G>A accounts for about 3.1% of genetically unsolved AR and sporadic cases of HSPs and ataxias (Minnerop *et al.*, 2017b; Gauquelin *et al.*, 2018; Di Donato *et al.*, 2021). In addition to a patient with compound heterozygous *POLR3A* variants (*POLR3A*: c.1909+22G>A and a protein-coding variant), we reported a first patient with homozygous intronic variant c.1909+22G>A. In addition to the potential held in the non-coding regions, the secondary allele can be presented in the form of CNVs which are not often detectable by ES. Our findings revealed three patients with a missense *SPG7* variant. Additional analysis of the patients using MLPA revealed *SPG7* CNVs. Thus, we highly recommend performing a gold standard method to detect CNVs especially in those HSP patients who are heterozygous AR gene carriers. We also suggest using several bioinformatic tools to detect potential CNVs from ES data.

Moving forward, as the use of NGS-based approaches become less costly, GS can be widely applied and overcome some of the limitations imposed by ES. Using GS, we identified a large deletion of *KIF1A* in one of the patients highlighting the significance of GS in improving genetic diagnosis in clinical settings. However, functional interpretation of the huge number of variants detected by GS remains an issue that would require transcriptomic data and further development of publicly available resources to address in order to investigate the consequence of these variants.

Before GS becomes more accessible in the genetic diagnosis of rare disorders, it should be mentioned that there are still more analyses that can be done on ES data to further

increase the genetic diagnostic yield of HSPs. For example, we found a rare synonymous *SPAST* variant in our cohort and then by literature review found further evidence regarding its pathogenicity. Similarly, we found a rare synonymous variant in *REEP2* but there are no functional studies that can confirm deleteriousness. Given the role of synonymous splice variants in rare Mendelian disorders (Li *et al.*, 2021), we suggest further studying synonymous variants during ES data analysis.

To further determine the genetic etiology of HSPs using ES data alone, we can take advantage of the locus heterogeneity of HSP to discover new genes by applying a network biology approach in combination with available genetics data from a large cohort. Utilizing similar approaches previously led to the identification of several HSP genes such as *NT5C2*, *AMPD2*, *ERLIN1* (Novarino *et al.*, 2014a). We were able to identify several new candidates in our unsolved cases that closely interact with known HSP proteins. For example, we identified a hemizygous *TCEAL1* variant inherited from a heterozygous unaffected mother in a single proband who was phenotypically matched with some patients from other cohorts (unpublished data). Similarly, a homozygous *RBSN* gene variant was identified in a Cree family and the clinical features were perfectly matched with patients from other cohorts carrying variants in the same gene (Paul *et al.*, 2022). We found two families in our cohort with compound heterozygous *SPTAN1* variants (Leveille *et al.*, 2019b). One of the variants p.(Ala858Ser) was carried by both unrelated French-Canadian probands. Following our study which was the first to report *SPTAN1* variants in HSP, several studies reported HSP patients with *SPTAN1* variants (Van de Vondel *et al.*, 2022; Xie *et al.*, 2022). We also found homozygous variants in two potentially new genes, *AP5B1* and *CAPNS2* in one Pakistani and one Iranian probands.

Although data from *in silico* tools as well as some previously published evidence support our finding regarding the association of AP5B1 and CAPNS2 with HSP, further functional experiments and identification of additional patients (probably from those two countries) are required for validation. The diagnostic odyssey for patients can be partially addressed by adding these new genes in panel sequencing where performing ES or GS methods are not feasible or costly.

In multiple HSP cohort studies where ES was performed, the screening was only focused on known HSP genes. However, HSPs share clinical and genetic overlap with multiple neurological and even non-neurological disorders. Some phenotypes of HSPs are in the borderline zone with ALS and PLS, spastic and cerebellar ataxias, neuropathies and leukodystrophies. Consequently, it is essential to improve diagnosis by identifying cases more objectively and minimizing subjective definitions, especially in the overlap zones to reduce the technical diagnostic challenges that result from insufficient expertise in the evolving field of neurogenetics.

Uncovering the genetic basis of HSPs is highly suggested - not only for research purposes, but also as a gold standard method in the clinical settings, as patients could in some cases present with spastic paraplegia, which is the predominant feature of HSPs, and end up showing additional symptoms that may result in a diagnosis of a distinct neurologic or non-neurologic disorder.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

Overall, the genetic heterogeneity of HSPs aligns with its clinical and molecular mechanisms heterogeneity. To identify the genetic causes of HSPs, the analyses should go beyond screening protein-coding variants in known HSP genes and simple Mendelian inheritance. We were able to identify the potential disease-causing variants in 68.2% of cases in this project which is the highest proportion of genetic diagnosis rate for HSPs thus far. The remaining 31.8% gap may be explained by several factors. For example, there are still new genes that need to be discovered likely in communities with high consanguinity rates since the majority of recently detected subtypes are AR-HSPs. Moreover, the contribution of epigenetic factors that was already demonstrated in PD, AD, ALS, and cerebellar ataxias (Chestnut *et al.*, 2011; Desplats *et al.*, 2011; Haertle *et al.*, 2019; Lardenoije *et al.*, 2019; Jia *et al.*, 2021) needs to be investigated. The environmental factors and stochasticity, which have not been studied in HSPs, may also play a role. This concept is highlighted by showing a broad range of manifestations among carriers of the same mutation within a family (Klebe *et al.*, 2015; Chrestian *et al.*, 2017b).

Our findings highlight the following challenges in the genetic diagnosis of HSPs: 1) the major genotypic and phenotypic overlap between HSPs and other spastic neurogenetic disorders or 2) disorders that resemble HSPs, 3) the large number of genes implicated in HSPs that are known or 4) being discovered rapidly in the NGS era, 5) the multiple inheritance modes of HSP genes, and 6) digenic inheritance of HSP.

The current results along with findings from other studies pinpoint axon-related pathways as underlying mechanisms in the etiopathogenesis of several common HSP subtypes as

well as the overlapping disorders. Thus, the common therapeutic targets could emerge probably with the alteration of the axon-related (projection, development, morphology) pathways. Additionally, identifying shared molecular mechanisms would help discover biomarkers that could be used to predict the progression of the disease and to elaborate treatment plans, thus improving the outcome. Biomarker identification may be less challenging in HSP subtypes associated with metabolic pathways such as SPG5 and SPG9. For future treatment options, besides a large collection of data from ancillary tests and clinical assessments such as the SPRS, utilizing combined molecular diagnostic approaches is necessary.

Collecting a genotypically- and phenotypically-similar cohort of a rare monogenic disorder with sufficient size could take many years, and therefore may be an inefficient method to support the newly discovered genes rapidly (Azzariti and Hamosh, 2020). Other challenges of rare disease research include the insufficiency of patient data and resources, as well as lack of definite treatments, a shortage of trained disease experts, which cause delayed diagnosis and management. These challenges could be partially overcome by adhering to Open Science principles such as sharing data and practices (Rubinstein *et al.*, 2020). Another solution to overcome the above-mentioned limitations could be developing tools or platforms that enable patients to share their own genotype, pedigree, and phenotype data in a consistent and well-informed manner. The available genomic resources and capacity that exist in developed countries can be linked to the available samples and data of consanguineous populations in less developed countries. As a result of the huge amount of data coming from different sources, machine learning and deep learning techniques are expected to be widely applied in a growing number of

methods to decode disease associations and patterns. However, these approaches could be of limited use due to the factors that affect interpretation of data, such as the training data quality. A strong resource for new gene discovery could be building of a publicly available database that could link the null variants and the functional evidence to the human phenotype. Moreover, developing platforms that connect researchers who share genotype or phenotype of interest would be beneficial for novel gene discovery.

CHAPTER 8: REFERENCES

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CHAPTER 8: APPENDICES

- **Chapter 2**

  Authorization of the co-authors

Re: Thesis requirement - authors agreement

Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Thu 7/7/2022 12:36 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

I agree

Ziv Gan-Or, MD, PhD, Assistant Professor
Director, Neurodegenerative Diseases Research Group
Neurogenomics and Precision Medicine (NAP-Med) lab
The Neuro (Montreal Neurological Institute-Hospital)
Department of Neurology & Neurosurgery
Department of Human Genetics
McGill University
1033 Pine Avenue West
Ludmer Pavilion, room 312
Montreal, QC, H3A 1A1
Lab: +1-514-398-5845
e-mail: ziv.gan-or@mcgill.ca
Website: www.nap-med.org

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 7, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Thesis requirement - authors agreement

Dear all,

I hope this email finds you well.

I am planning to submit my thesis which is manuscript-based and includes 4 chapters. The three chapters are previously published papers that I am a first or co-first author, and one of them is an unpublished manuscript. Please find the unpublished manuscript in the attachment. I also added the title page below.

According to the rules of McGill University, it is required to get the authorization of all co-authors to use a non-published manuscript in the thesis. I would be very grateful if you could confirm your agreement to use the attached manuscript in my thesis by answering this email at your earliest convenience?

Of course, we will send the manuscript to the authors before submitting it to any journal or medRxiv, and additional changes may be applied in the future.

Many thanks in advance for your prompt response.

The genetic landscape of hereditary spastic paraplegia in Canada

Mehrdad A Estiar[1,2], Eric Yu[1,2], Parizad Varghaei[2,3], Etienne Leveille[4], Setareh Ashtiani[5], Lauren Brady[6], Farnaz Asayesh[2], Nicolas Dupré[7], Kym M. Boycott[8], Oksana Suchowersky[9], Grace Yoon[10], Mark Tarnopolsky[11], Jean François Trempe[12], Guy A. Rouleau[1,2,13*], Ziv Gan-Or[1,2,13*]

1. Department of Human Genetics, McGill University, Montréal, Quebec, Canada
2. Montreal Neurological Institute and Hospital, McGill University, Montréal, Quebec, Canada
3. Division of Experimental Medicine, Department of Medicine, McGill University, Montréal, Quebec, Canada
4. Faculty of Medicine, McGill University, Montréal, Quebec, Canada
5. Alberta Children's Hospital, Medical Genetics, Calgary, Alberta, Canada.
6. Genetic Counselling Program, Hamilton Health Sciences, Hamilton, ON, Canada.
7. Division of Neurosciences, CHU de Québec, Université Laval, Québec City, QC, Canada
8. Department of Genetics, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada
9. Department of Medicine, Division of Neurology, University of Alberta, Edmonton, Alberta, Canada
10. Division of Neurology, Department of Paediatrics, University of Toronto, The Hospital for Sick Children, Toronto, Canada
11. Department of Pediatrics and Medicine, McMaster University, Hamilton, Canada
12. Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada
13. Department of Neurology and Neurosurgery, McGill University, Montréal, Quebec, Canada

Correspondence to: Guy A. Rouleau, Ziv Gan-Or

Best regards,

Mehrdad Asghari Estiar
PhD Candidate | McGill University
Department of Human Genetics
The Neuro (Montreal Neurological Institute-Hospital)
Phone: +1-514-772-8765
E-mail: mehrdad.a.estiar@mail.mcgill.ca

RE: Thesis requirement - authors agreement

Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>
Thu 7/7/2022 6:25 PM

To: 'Grace Yoon' <grace.yoon@utoronto.ca>;Tarnopolsky, Mark <tarnopol@mcmaster.ca>;Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>

Cc: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>;Eric Yu <eric.yu@mail.mcgill.ca>;Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>;Leveille, Etienne <etienne.leveille@yale.edu>;Brady Lauren <bradyla@HHSC.CA>;Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>;Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>;Boycott, Kym <KBoycott@cheo.on.ca>;Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>;Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>;Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>

Hi Mehrdad,

Absolutely. Hope the submission goes well!

Setareh

Setareh Ashtiani, MSc, CCGC
Certified Genetic Counsellor
Phone: 587-774-4757
Email: setareh.ashtiani@ahs.ca

From: Grace Yoon <grace.yoon@utoronto.ca>
Sent: Thursday, July 7, 2022 2:39 PM
To: Tarnopolsky, Mark <tarnopol@mcmaster.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>
Cc: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>; Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Re: Thesis requirement - authors agreement

This is fine with me as well!

Grace

From: Tarnopolsky, Mark <tarnopol@mcmaster.ca>
Sent: July 7, 2022 2:40 PM
To: Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>
Cc: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>; Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@hhsc.ca>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; Grace Yoon

<grace.yoon@utoronto.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Re: Thesis requirement - authors agreement

Agree

Sent from my iPhone

On Jul 7, 2022, at 17:19, Guy Rouleau, Dr. <guy.rouleau@mcgill.ca> wrote:

OK for me

Guy Rouleau, OC, OQ, MD, PhD, FRCPC, FRSC
Director, The Neuro (Montreal Neurological Institute-Hospital)
Chair, Department of Neurology and Neurosurgery
McGill University
Chair, Department of Neuroscience
McGill University Health Center

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 7, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca; tarnopol@mcmaster.ca; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Thesis requirement - authors agreement

Dear all,

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Many thanks in advance for your prompt response.

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Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>
Thu 7/7/2022 7:37 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

I agree! Good luck :)

Parizad

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 7, 2022 12:35:18 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
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Many thanks in advance for your prompt response.

The genetic landscape of hereditary spastic paraplegia in Canada

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1. Department of Human Genetics, McGill University, Montréal, Quebec, Canada
2. Montreal Neurological Institute and Hospital, McGill University, Montréal, Quebec, Canada
3. Division of Experimental Medicine, Department of Medicine, McGill University, Montréal, Quebec, Canada
4. Faculty of Medicine, McGill University, Montréal, Quebec, Canada

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11. Department of Pediatrics and Medicine, McMaster University, Hamilton, Canada
12. Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada
13. Department of Neurology and Neurosurgery, McGill University, Montréal, Quebec, Canada

Correspondence to: Guy A. Rouleau, Ziv Gan-Or

Best regards,

Mehrdad Asghari Estiar
PhD Candidate | McGill University
Department of Human Genetics
The Neuro (Montreal Neurological Institute-Hospital)
Phone: +1-514-772-8765
E-mail: mehrdad.a.estiar@mail.mcgill.ca

Re: Thesis requirement - authors agreement

Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>
Thu 7/7/2022 1:18 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

Agreed

Nicolas Dupré MD MSc FRCP FAAN
Neurologue / Neurologist
CHU de Québec - Université Laval
Professeur Titulaire / Full professor
Faculté de médecine, Université Laval
nicolas.dupre.med@ssss.gouv.qc.ca

De : Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Envoyé : Thursday, July 7, 2022 12:35:18 PM
À : Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Objet : Thesis requirement - authors agreement

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E-mail: mehrdad.a.estiar@mail.mcgill.ca

Re: Thesis requirement - authors agreement

Tarnopolsky, Mark <tarnopol@mcmaster.ca>
Thu 7/7/2022 2:40 PM

To: Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>

Cc: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>;Eric Yu <eric.yu@mail.mcgill.ca>;Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>;Leveille, Etienne <etienne.leveille@yale.edu>;Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>;Brady Lauren <bradyla@hhsc.ca>;Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>;Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>;Boycott, Kym <KBoycott@cheo.on.ca>;Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>;grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>;Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>;Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>

Agree

Sent from my iPhone

> On Jul 7, 2022, at 17:19, Guy Rouleau, Dr. <guy.rouleau@mcgill.ca> wrote:
>
> OK for me
>
> Guy Rouleau, OC, OQ, MD, PhD, FRCPC, FRSC
> Director, The Neuro (Montreal Neurological Institute-Hospital)
> Chair, Department of Neurology and Neurosurgery
> McGill University
> Chair, Department of Neuroscience
> McGill University Health Center
>
> ---
>
> **From:** Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
> **Sent:** Thursday, July 7, 2022 12:35 PM
> **To:** Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca; tarnopol@mcmaster.ca; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
> **Subject:** Thesis requirement - authors agreement
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Best regards,

Mehrdad Asghari Estiar

PhD Candidate | McGill University

Department of Human Genetics

The Neuro (Montreal Neurological Institute-Hospital)

Phone: +1-514-772-8765

E-mail: mehrdad.a.estiar@mail.mcgill.ca

RE: Thesis requirement - authors agreement

Brady Lauren <bradyla@HHSC.CA>
Thu 7/7/2022 4:07 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

Hi Mehrdad,

I agree. You can change my affiliation to be the same as Dr. Tarnopolsky's

Lauren Brady, MSc, CCGC, CGC
Certified Genetic Counsellor
2H Neuromuscular and Neurometabolic Clinic
McMaster University Medical Centre
PHONE: (905) 521-2100 x76932
FAX: (905) 521-2638

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 07, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym (Children's Hospital Of Eastern Ontario) <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca; Tarnopolsky, Mark (McMaster) <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Thesis requirement - authors agreement

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E-mail: mehrdad.a.estiar@mail.mcgill.ca

Re: Thesis requirement - authors agreement

Boycott, Kym <KBoycott@cheo.on.ca>
Thu 7/7/2022 1:43 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

EXTERNAL MAIL*
agree.

Kym Boycott, MD, PhD, FRCPC, FCCMG
Clinician Scientist, CHEO Research Institute
Professor of Pediatrics, University of Ottawa

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 7, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
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Re: Thesis requirement - authors agreement

Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>
Fri 7/8/2022 12:24 AM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Cc: Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>

Dear Mehrdad,

I agree. Good luck!

Best
JF

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Date: Thursday, July 7, 2022 at 6:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>, Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>, "Leveille, Etienne" <etienne.leveille@yale.edu>, Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>, Brady Lauren <bradyla@HHSC.CA>, "Farnaz Asayesh, Mrs" <farnaz.asayesh@mcgill.ca>, "Nicolas Dupre (CHU-MED)" <nicolas.dupre.med@ssss.gouv.qc.ca>, "Boycott, Kym" <KBoycott@cheo.on.ca>, Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>, "grace.yoon@utoronto.ca" <grace.yoon@utoronto.ca>, "tarnopol@mcmaster.ca" <tarnopol@mcmaster.ca>, "Jean-Francois Trempe, Prof." <jeanfrancois.trempe@mcgill.ca>, "Guy Rouleau, Dr." <guy.rouleau@mcgill.ca>, "Ziv Gan-Or, Dr." <ziv.gan-or@mcgill.ca>
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E-mail: mehrdad.a.estiar@mail.mcgill.ca

Re: Thesis requirement - authors agreement

Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>
Thu 7/7/2022 12:44 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

Hello Mehrdad,

I'm agreed.

Best,
Farnaz

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 7, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
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1. Department of Human Genetics, McGill University, Montréal, Quebec, Canada

2. Montreal Neurological Institute and Hospital, McGill University, Montréal, Quebec, Canada
3. Division of Experimental Medicine, Department of Medicine, McGill University, Montréal, Quebec, Canada
4. Faculty of Medicine, McGill University, Montréal, Quebec, Canada
5. Alberta Children's Hospital, Medical Genetics, Calgary, Alberta, Canada.
6. Genetic Counselling Program, Hamilton Health Sciences, Hamilton, ON, Canada.
7. Division of Neurosciences, CHU de Québec, Université Laval, Québec City, QC, Canada
8. Department of Genetics, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada
9. Department of Medicine, Division of Neurology, University of Alberta, Edmonton, Alberta, Canada
10. Division of Neurology, Department of Paediatrics, University of Toronto, The Hospital for Sick Children, Toronto, Canada
11. Department of Pediatrics and Medicine, McMaster University, Hamilton, Canada
12. Department of Pharmacology & Therapeutics, McGill University, Montréal, Québec, Canada
13. Department of Neurology and Neurosurgery, McGill University, Montréal, Quebec, Canada

Correspondence to: Guy A. Rouleau, Ziv Gan-Or

Best regards,

Mehrdad Asghari Estiar
PhD Candidate | McGill University
Department of Human Genetics
The Neuro (Montreal Neurological Institute-Hospital)
Phone: +1-514-772-8765
E-mail: mehrdad.a.estiar@mail.mcgill.ca

Re: Thesis requirement - authors agreement

Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>

Fri 7/8/2022 11:10 PM

To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

Yes, I approve.

Oksana Suchowersky, MD, FRCPC, FCCMG
Professor of Medicine (Neurology), Medical Genetics, and Pediatrics,
University of Alberta
7-112Q Clinical Sciences Building
11350 83 Ave
Edmonton Alberta Canada T6G 2G3
Phone: (780) 248-5418
Fax: (780) 248-1807

Re: Thesis requirement - authors agreement

Leveille, Etienne <etienne.leveille@yale.edu>
Thu 7/7/2022 1:35 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>

Hi Mehrdad,

Its all good for me.

Best,

Etienne

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: July 7, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Thesis requirement - authors agreement

Dear all,

I hope this email finds you well.

I am planning to submit my thesis which is manuscript-based and includes 4 chapters. The three chapters are previously published papers that I am a first or co-first author, and one of them is an unpublished manuscript. Please find the unpublished manuscript in the attachment. I also added the title page below.

According to the rules of McGill University, it is required to get the authorization of all co-authors to use a non-published manuscript in the thesis. I would be very grateful if you could confirm your agreement to use the attached manuscript in my thesis by answering this email at your earliest convenience?

Of course, we will send the manuscript to the authors before submitting it to any journal or medRxiv, and additional changes may be applied in the future.

Many thanks in advance for your prompt response.

The genetic landscape of hereditary spastic paraplegia in Canada

Mehrdad A Estiar[1,2], Eric Yu[1,2], Parizad Varghaei[2,3], Etienne Leveille[4], Setareh Ashtiani[5], Lauren Brady[6], Farnaz Asayesh[2], Nicolas Dupré[7], Kym M. Boycott[8], Oksana Suchowersky[9], Grace Yoon[10], Mark Tarnopolsky[11], Jean François Trempe[12], Guy A. Rouleau[1,2,13*], Ziv Gan-Or[1,2,13*]

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Phone: +1-514-772-8765
E-mail: mehrdad.a.estiar@mail.mcgill.ca

Re: Thesis requirement - authors agreement

Eric Yu <eric.yu@mail.mcgill.ca>
Thu 7/7/2022 4:03 PM
To: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Agree

Eric Yu
PhD Student | McGill University
Department of Human Genetics
The Neuro (Montreal Neurological Institute-Hospital)

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
Sent: Thursday, July 7, 2022 12:35 PM
To: Eric Yu <eric.yu@mail.mcgill.ca>; Parizad Varghaei <parizad.varghaei@mail.mcgill.ca>; Leveille, Etienne <etienne.leveille@yale.edu>; Setareh Ashtiani <Setareh.Ashtiani@albertahealthservices.ca>; Brady Lauren <bradyla@HHSC.CA>; Farnaz Asayesh, Mrs <farnaz.asayesh@mcgill.ca>; Nicolas Dupre (CHU-MED) <nicolas.dupre.med@ssss.gouv.qc.ca>; Boycott, Kym <KBoycott@cheo.on.ca>; Oksana Suchowersky <Oksana.Suchowersky@albertahealthservices.ca>; grace.yoon@utoronto.ca <grace.yoon@utoronto.ca>; tarnopol@mcmaster.ca <tarnopol@mcmaster.ca>; Jean-Francois Trempe, Prof. <jeanfrancois.trempe@mcgill.ca>; Guy Rouleau, Dr. <guy.rouleau@mcgill.ca>; Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>
Subject: Thesis requirement - authors agreement

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Many thanks in advance for your prompt response.

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PhD Candidate | McGill University
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Phone: +1-514-772-8765
E-mail: mehrdad.a.estiar@mail.mcgill.ca

Supplementary Table 2.1. Characteristics of control individuals.

Available upon request. The same table as Table S2 in:

https://movementdisorders.onlinelibrary.wiley.com/doi/10.1002/mds.28528

Supplementary Table 2.2. Clinical characteristics of HSP probands.

Given the large size of the table, available upon request.

Supplementary Table 2.3. The list of genes, frequency and type of variants identified in the HSP patients.

Disease	Inheritance	Gene	Frequency of variants	Missense_ Synonymous	LoF	In-frame Indels	Notable findings
SPG9A	AD	*ALDH18A1*	1	1	0	0	The variant occurred in Glutamate 5-kinase domain (G5K) which facilitates catalysation of glutamate to gamma-glutamyl phosphate.
SPG52	AR	*AP4S1*	1	0	1	0	The variant activates a cryptic splice donor site.
SPG3A	AD	*ATL1*	11	7	1	3	p.(Met347Thr) is the most common variant in our SPG3A patients. Five missense variants occurred in GTPase domain that is induced by interferon-gamma
SPG78	AR	*ATP13A2*	3	2	1	0	p.(Arg709Thr) occurred within the hydrolase domain which is critical for the catalytic activity. The variant may affect splicing and possibly result in nonsense mediated decay. The variant p.(Gly720Trp) could unfold the N-domain of ATP13A2.
SPG	AD	*BSCL2*	1	1	0	0	(p.Ser154Leu) occurred in Seipin which is a cell-autonomous regulator of lipolysis essential for adipocyte differentiation
SPG43	AR	*C19orf12*	1	0	1	0	This is the second HSP family with *C19orf12* variant that has been reported so far
SPG76	AR	*CAPN1*	4	0	4	0	All variants are loss-of-function that result in a dysfunctional protein without "Peptidase C2" and "EF-hand" domains
SPG73	AD	*CPT1C*	1	0	1	0	This is the third HSP family with *CPT1C* variant that has been reported
SPG5A	AR	*CYP7B1*	9	5	4	0	(p.Tyr275Ter) is the most common variant that truncate the full length protein from 506 aa to 275 aa
SPG18	AR	*ERLIN2*	1	1	0	0	The variant reside in the last residue of "oligomerization" domain
SPG35	AR	*FA2H*	4	3	1	0	(p.Tyr231His) which occurred in "fatty acid hydroxylase" domain, was carried by 2/3 patients with *FA2H* variants. Both patients are French-Canadian. (p.Gly217Arg) is located in transmembrane domain
SPG77	AR	*FARS2*	2	2	0	0	Both variants occurred in "anticodon-binding" domain
SPG46	AR	*GBA2*	1	0	1	0	This variant gives rise to the synthesis of truncated GBA2 protein
SPG13	AD	*HSPD1*	1	1	0	0	The variant occurrd in apical domain involved in substrate binding
SPG	AD	*KIDINS220*	2	2	0	0	Both variants occurred in the potential phosphorylation sites (Y1383C; Score=0.564, phosphorylated by EGFR & S672F; Score=0.559; phosphorylated by PKA) according to NetPhos 3.1
SPG30	AD/AR	*KIF1A*	6	5	1	0	Both heterozygous and homozygous variants were seen. All but one variants occurred in Kinesin motor domain
SPG10	AD	*KIF5A*	2	1	1	0	The frameshift deletion gives rise a protein without "coiled coil" and "globular" domains which are important for cargo binding and heavy chain dimerization. The missense variant occurred in globular domain
SPG75	AR	*MAG*	2	2	0	0	Both variants occurred in Immunoglobulin-like domains
SPG70	AD/AR	*MARS1*	3	3	0	0	_
SPG6	AD	*NIPA1*	2	2	0	0	Both families harbored the same variant which occurred in "Multidrug resistance efflux transporter EmrE" domain
SPG45	AR	*NT5C2*	1	1	0	0	_
SPG2	AD	*PLP1*	5	2	3	0	(p.Thr250Ser) occurred in transmembrane region and (p.Arg127Thr) is located in Cytoplasmic domain
SPG39	AR	*PNPLA6*	4	2	2	0	Both missense variants occurred in "patatins and phospholipases" domain
SPG31	AD	*REEP1*	8	3	5	0	The missense variants localized in three domains; transmembrane domains 1, 2, and a "deleted in polyposis" domain
SPG22	XL	*SLC16A2*	1	1	0	0	The variant occurred in MFS general substrate transporter like domain
SPG4	AD	*SPAST*	65	38_2	23	2	A case with homozygous variant, a family with compound heterozygous variants, and three patients with *de novo* variants. Among the missense variants, 75% were clustered in AAA cassette, while LoF variants were more evenly distributed across gene
SPG11	AR	*SPG11*	31	3	27	1	27/31 variants are LoF. The second most common variant in our cohort is (p.Lys2200Ter) and all 7 carriers are French-Canadian.
SPG7	AR	*SPG7*	65	42	23	0	c.1529C>T is the most frequent variant in our study that was detected in 21 SPG7 families.
SPG49	AR	*TECPR2*	1	0	1	0	This is the third study reporting *TECPR2* variants in HSPs
SPG80	AD	*UBAP1*	2	1	1	0	_
SPG8	AD	*WASHC5*	5	5	0	0	_
SPG15	AR	*ZFYVE26*	3	0	3	0	_
SPG33	AD	*ZFYVE27*	1	1	0	0	The variant occurred in Cytoplasmic domain
Adrenomyeloneuropathy	XLR	*ABCD1*	3	1	2	0	The missense variant occurred in ABC transporter type 1 transmembrane domain that is involved in the export and import of a wide variety of substrates
Polyneuropathy	AR	*ABHD12*	1	0	1	0	This is the first report of *ABHD12* variant in HSPs
Spinocerebellar ataxia 28	AD	*AFG3L2*	2	2	0	0	One patient carried a heterozygous *SPG7* variant along with *AFG3L2* variant. Both *AFG3L2* and *SPG7* are paralogue genes (p.Ile705Thr) and (p.Gly419Ser) occurred in the peptidase_M41 and ATPase domains of AFG3L2 respectively.
Amyotrophic lateral sclerosis 2	AR	*ALS2*	2	1	1	0	The missense variant occurred in VPS9 domain that is essential for ALS2 function.
Spinocerebellar ataxia 10	AR	*ANO10*	1	1	0	0	This is the first report of *ANO10* in HSPs. The variant occurred in Cytoplasmic domain of ANO10.
Neuropathy, hereditary sensor	AD	*ATL3*	1	1	0	0	This is the first report of *ATL3* in HSPs. The variant occurred in GTPase domain that is induced by interferon-gamma.

Disease	Inheritance	Gene	Frequency of variants	Missense_ Synonymous	LoF	In-frame Indels	Notable findings
Charcot-Marie-Tooth disease, a	AD	*ATP1A1*	1	1	0	0	The variant occurred in Cation-transporting P-type ATPase that use ATP hydrolysis to drive the transport of protons across a membrane.
Spinocerebellar ataxia	XLR	*ATP2B3*	2	2	0	0	This is the first report of *ATP2B3* variants in HSPs. (p.Ala822Thr) occurred in P-type ATPase, haloacid dehalogenase domain that use ATP hydrolysis to drive the transport of protons across a membrane.
Spinal muscular atrophy, distal	XLR	*ATP7A*	1	1	0	0	This is the first report of *ATP7A* variants in HSPs. The variant occurred in heavy metal-associated domain
Cerebellar ataxia	AD	*CAMTA1*	1	1	0	0	This is the first report of *CAMTA1* variants in HSPs.
Bethlem myopathy 1	AD/AR	*COL6A1*	1	1	0	0	This is the first report of *COL6A1* variants in HSPs. The variant occurred in collagen triple helix repeat.
Charcot-Marie-Tooth disease, a	AD	*DNM2*	1	1	0	0	The variant occurred in dynamin stalk domain which is involved in membrane remodelling and is critical for endocytic membrane fission.
Charcot-Marie-Tooth disease, a	AD	*DYNC1H1*	2	2	0	0	(p.Arg3219His) occurred in dynein heavy chain coiled coil stalk domain
Dystonia, DOPA-responsive	AD/AR	*GCH1*	2	1	0	1	(p.Val205Glu) and (p.Ser77_Leu82del) are probably affecting the proper homodecamerization of GCH1, preventing the assembly of the three subunit-formed active sites.
Developmental and epileptic e	AD	KCNA2	1	1	0	0	The variant occurred in cytoplasmic domain of KCNA2
Charcot-Marie-Tooth disease, a	AD	*NEFH*	1	1	0	0	This is the first report of *NEFH* variants in HSPs. The variant occurred in neurofilament triplet protein of NEFH
Charcot-Marie-Tooth disease, t	AD	*NEFL*	1	1	0	0	The variant is exonic splicing that occurred in NEFL rod domain
Basal ganglia calcification, idio	AD	*PDGFB*	1	0	1	0	This is the first report of *PDGFB* variants in HSPs.
Leukodystrophy, hypomyelina	AR	*POLR3A*	3	3	0	0	(p.Arg1069Trp) occurred in DNA-directed RNA polymerase III subunit RPC1 domain
Spastic ataxia, Charlevoix-Sagu	AR	*SACS*	10	7	3	0	Two missense variants occurred in histidine kinase/HSP90-like ATPase superfamily
Neuropathy, hereditary sensor	AD	*SCN11A*	1	1	0	0	This is the first report of *SCN11A* variant in HSPs. The variant occurred in non-cytoplasmic domain
Amyotrophic lateral sclerosis 4	AD	*SETX*	4	4	0	0	_
Spinocerebellar ataxia 5	AD	*SPTBN2*	1	1	0	0	The variant occurred in Spectrin/alpha-actinin
Spinocerebellar ataxia, autoso	AR	*SYNE1*	1	0	1	0	_
Leukodystrophy, hypomyelina	AD	*TUBB4A*	1	1	0	0	The variant occurred in tubulin C domain
Spastic ataxia 1	AD	*VAMP1*	3	0	3	0	_
Achalasia-addisonianism-alacr	AR	*AAAS*	2	0	2	0	_
Developmental and epileptic e	AD	*CACNA1E*	1	1	0	0	This is the first report of *CACNA1E* variant in HSPs. The variant occurred in cytoplasmic domain
Brain small vessel disease 2 (B	AD	*COL4A2*	1	1	0	0	This is the first report of *COL4A2* variant in HSPs. The variant occurred in collagen triple helix repeat
Bethlem myopathy 1	AD/AR	*COL6A1*	1	1	0	0	This is the first report of *COL6A1* variant in HSPs. The variant occurred in von Willebrand factor, type A domain
Leukoencephalopathy, diffuse	AD	*CSF1R*	1	1	0	0	This is the first report of *CSF1R* variant in HSPs. The variants occurred in Immunoglobulin-like domain
Neurodevelopmental disorder	AD	*CTNNB1*	1	0	1	0	_
Encephalopathy, lethal, due to	AD/AR	*DNM1L*	1	0	1	0	_
Krabbe disease	AR	*GALC*	2	1	1	0	The variant occurred in glycoside hydrolase domain
Chediak-Higashi syndrome	AR	*LYST*	2	2	0	0	(p.Pro3224Ala) occurred in the BEACH domain implicated in membrane trafficking
Mucolipidosis IV	AR	*MCOLN1*	2	1	1	0	This is the first report of *MCOLN1* variant in HSPs.
Infantile neuroaxonal dystroph	AR	*PLA2G6*	2	2	0	0	Both variants occurred in phospholipase domain
Basal ganglia calcification, idiop	AD	*SLC20A2*	2	2	0	0	This is the first report of *SLC20A2* variant in HSPs. (p.Gly73Val) and (p.Gly512Ser) occurred in cytoplasmic and non-cytoplasmic domains respectively
GLUT1 deficiency syndrome	AD	*SLC2A1*	1	1	0	0	The variant occurred in major facilitator superfamily domain
Hartnup disorder	AR	*SLC6A19*	1	1	0	0	The variant occurred in sodium:neurotransmitter symporter, taurine domain
Revesz syndrome	AD	*TINF2*	2	1	1	0	This is the first report of *TINF2* variant in HSPs. The variant occurred in non-cytoplasmic domain.
New gene	AR	*AP5B1*	1	1	0	0	Explained in the text
New gene	AR	*CAPNS2*	1	1	0	0	Explained in the text
New gene	AR	*RBSN*	1	1	0	0	Explained in the text
New gene	AR	*SPTAN1*	4	4	0	0	Explained in the text
New gene	XL	*TCEAL1*	1	1	0	0	Explained in the text
New gene	AD	*KPNA3*	2	2	0	0	Explained in the text

Supplementary Table 2.4. Gene Ontology (GO) term enrichment with respect to Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). *P* values were corrected for multiple testing using the Benjamini−Hochberg false discovery rate. Adjusted *P* values show the significance of enrichment with the threshold of $P < 0.05$. The list of genes carrying variants that were identified in this study, were included in the analysis. The enrichment results of known HSP genes were compared with the results of genes implicated in overlapping disorders, and known HSP genes versus genes involved in mimicking disorders. Only shared pathways are shown.

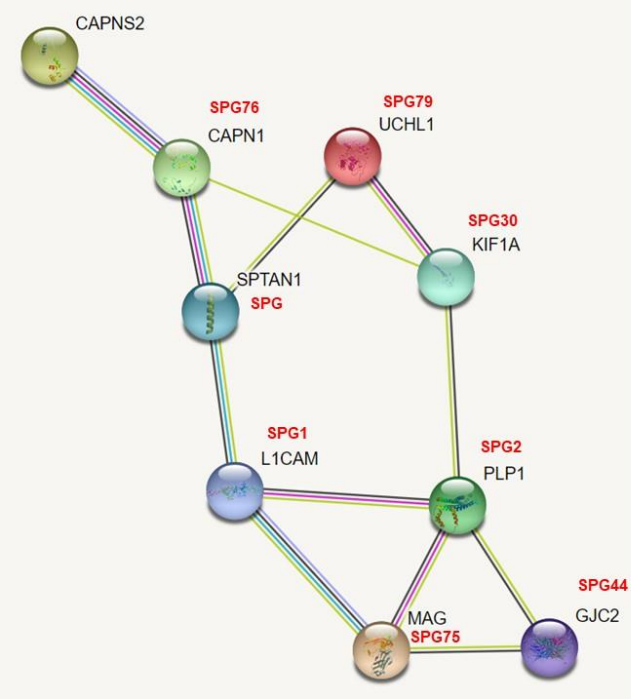
Gene Ontology enrichment analysis on known HSP proteins				
source	term_name	term_id	adjusted_	intersections
GO:MF	adenyl ribonucleotide binding	GO:0032559	0.001753	ALDH18A1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	ATP binding	GO:0005524	0.001753	ALDH18A1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	purine ribonucleotide binding	GO:0032555	0.001753	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	purine nucleotide binding	GO:0017076	0.001753	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	nucleoside-triphosphatase activity	GO:0017111	0.001753	ATL1,ATP13A2,HSPD1,KIF1A,KIF5A,SPAST,SPG7
GO:MF	carbohydrate derivative binding	GO:0097367	0.001753	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MAG,MARS1,NT5C2,SPAST,SPG7
GO:MF	ATP hydrolysis activity	GO:0016887	0.001753	ATP13A2,HSPD1,KIF1A,KIF5A,SPAST,SPG7
GO:MF	ribonucleotide binding	GO:0032553	0.001753	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	purine ribonucleoside triphosphate binding	GO:0035639	0.001753	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	adenyl nucleotide binding	GO:0030554	0.001753	ALDH18A1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	pyrophosphatase activity	GO:0016462	0.001934	ATL1,ATP13A2,HSPD1,KIF1A,KIF5A,SPAST,SPG7
GO:MF	hydrolase activity, acting on acid anhydrides	GO:0016817	0.001934	ATL1,ATP13A2,HSPD1,KIF1A,KIF5A,SPAST,SPG7
GO:MF	hydrolase activity, acting on acid anhydrides, in phosphorus-cont	GO:0016818	0.001934	ATL1,ATP13A2,HSPD1,KIF1A,KIF5A,SPAST,SPG7
GO:MF	anion binding	GO:0043168	0.002004	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MAG,MARS1,NT5C2,SPAST,SPG7
GO:MF	ATP-dependent activity	GO:0140657	0.002178	ATP13A2,HSPD1,KIF1A,KIF5A,SPAST,SPG7
GO:MF	small molecule binding	GO:0036094	0.002562	ALDH18A1,ATL1,ATP13A2,ERLIN2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	nucleoside phosphate binding	GO:1901265	0.002801	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	nucleotide binding	GO:0000166	0.002801	ALDH18A1,ATL1,ATP13A2,FARS2,HSPD1,KIF1A,KIF5A,MARS1,NT5C2,SPAST,SPG7
GO:MF	hydrolase activity	GO:0016787	0.007241	ATL1,ATP13A2,CAPN1,GBA2,HSPD1,KIF1A,KIF5A,NT5C2,PNPLA6,SPAST,SPG7
GO:MF	ATP-dependent peptidase activity	GO:0004176	0.037554	SPG7
GO:BP	axo-dendritic transport	GO:0008088	0.000146	KIF1A,KIF5A,SPAST,SPG11,SPG7
GO:BP	cell morphogenesis involved in neuron differentiation	GO:0048667	0.000401	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	axonal transport	GO:0098930	0.000464	KIF1A,KIF5A,SPAST,SPG7
GO:BP	cell projection morphogenesis	GO:0048858	0.000482	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	neuron development	GO:0048666	0.000482	ATL1,GBA2,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	transport along microtubule	GO:0010970	0.000482	KIF1A,KIF5A,SPAST,SPG11,SPG7
GO:BP	neuron projection morphogenesis	GO:0048812	0.000482	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	plasma membrane bounded cell projection morphogenesis	GO:0120039	0.000482	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	cell part morphogenesis	GO:0032990	0.000543	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	axon development	GO:0061564	0.000604	ATL1,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	cell morphogenesis involved in differentiation	GO:0000904	0.000756	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	cytoskeleton-dependent intracellular transport	GO:0030705	0.000756	KIF1A,KIF5A,SPAST,SPG11,SPG7
GO:BP	neuron projection development	GO:0031175	0.000756	ATL1,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	microtubule-based transport	GO:0099111	0.000776	KIF1A,KIF5A,SPAST,SPG11,SPG7
GO:BP	cellular component morphogenesis	GO:0032989	0.000906	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	neurogenesis	GO:0022008	0.001249	ATL1,FA2H,GBA2,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	neuron differentiation	GO:0030182	0.001474	ATL1,GBA2,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	membrane organization	GO:0061024	0.001539	ATL1,ATP13A2,FA2H,GBA2,REEP1,SPAST,SPG11,SPG7
GO:BP	generation of neurons	GO:0048699	0.001886	ATL1,GBA2,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	cell development	GO:0048468	0.001886	ATL1,FA2H,GBA2,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,WASHC5,ZFYVE27
GO:BP	axonogenesis	GO:0007409	0.002153	ATL1,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	cell morphogenesis	GO:0000902	0.004073	ATL1,KIDINS220,KIF1A,KIF5A,MAG,SPAST,SPG11,ZFYVE27
GO:BP	nervous system development	GO:0007399	0.005081	ATL1,FA2H,GBA2,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,SPG7,ZFYVE27
GO:BP	endomembrane system organization	GO:0010256	0.005081	ATL1,FA2H,REEP1,SPAST,WASHC5,ZFYVE27
GO:BP	regulation of cellular component size	GO:0032535	0.00557	ATP13A2,GBA2,MAG,WASHC5,ZFYVE27
GO:BP	organelle transport along microtubule	GO:0072384	0.007092	KIF1A,KIF5A,SPAST
GO:BP	plasma membrane bounded cell projection organization	GO:0120036	0.0075	ATL1,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	microtubule-based movement	GO:0007018	0.007763	KIF1A,KIF5A,SPAST,SPG11,SPG7
GO:BP	establishment of localization	GO:0051234	0.008089	AP4S1,ATP13A2,CAPN1,HSPD1,KIF1A,KIF5A,NIPA1,PLP1,REEP1,SLC16A2,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE27
GO:BP	cell projection organization	GO:0030030	0.008317	ATL1,KIDINS220,KIF1A,KIF5A,MAG,PLP1,SPAST,SPG11,ZFYVE27
GO:BP	establishment of organelle localization	GO:0051656	0.008317	ATP13A2,KIF1A,KIF5A,SPAST,SPG11
GO:BP	retrograde axonal transport	GO:0008090	0.00947	KIF1A,KIF5A
GO:BP	regulation of biological quality	GO:0065008	0.016076	ATP13A2,BSCL2,C19ORF12,CPT1C,CYP7B1,FA2H,GBA2,HSPD1,KIF1A,MAG,SLC16A2,SPG7,WASHC5,ZFYVE27
GO:BP	transport	GO:0006810	0.016106	AP4S1,ATP13A2,CAPN1,HSPD1,KIF1A,KIF5A,NIPA1,PLP1,SLC16A2,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE27
GO:BP	regulation of anatomical structure size	GO:0090066	0.016423	ATP13A2,GBA2,MAG,WASHC5,ZFYVE27
GO:BP	localization	GO:0051179	0.019105	AP4S1,ATP13A2,BSCL2,CAPN1,CYP7B1,HSPD1,KIF1A,KIF5A,NIPA1,PLP1,REEP1,SLC16A2,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE27
GO:BP	organelle localization	GO:0051640	0.021727	ATP13A2,KIF1A,KIF5A,SPAST,SPG11
GO:BP	cellular localization	GO:0051641	0.021727	AP4S1,ATP13A2,HSPD1,KIF1A,KIF5A,REEP1,SPAST,SPG11,SPG7,TECPR2,WASHC5,ZFYVE27
GO:CC	organelle membrane	GO:0031090	5.39E-07	ALDH18A1,AP4S1,ATL1,ATP13A2,BSCL2,C19ORF12,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIF1A,PNPLA6,REEP1,SPAST,SPG11,SPG7,UBAP1,ZFYVE26,ZFYVE27
GO:CC	endomembrane system	GO:0012505	3.88E-06	AP4S1,ATL1,ATP13A2,BSCL2,C19ORF12,CAPN1,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIDINS220,KIF1A,NIPA1,PNPLA6,REEP1,SPAST,UBAP1,WASHC5,ZFYVE26,ZFYVE27
GO:CC	axon	GO:0030424	1.66E-05	ATL1,CPT1C,KIF1A,KIF5A,MAG,SPAST,SPG11,SPG7,ZFYVE27
GO:CC	membrane	GO:0016020	1.78E-05	ALDH18A1,AP4S1,ATL1,ATP13A2,BSCL2,C19ORF12,CAPN1,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIDINS220,KIF1A,KIF5A,MAG,MARS1,NIPA1,PLP1,PNPLA6,REEP1,SLC16A2,SPAST,SPG11,SPG7,UBAP1,ZFYVE26,ZFYVE27
GO:CC	organelle subcompartment	GO:0031984	2.17E-05	AP4S1,ATL1,BSCL2,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,PNPLA6,REEP1,SPAST,ZFYVE27
GO:CC	cytoplasm	GO:0005737	4.04E-05	ALDH18A1,AP4S1,ATL1,ATP13A2,BSCL2,C19ORF12,CAPN1,CPT1C,CYP7B1,ERLIN2,FA2H,FARS2,GBA2,HSPD1,KIDINS220,KIF1A,KIF5A,MAG,MARS1,NIPA1,NT5C2,PNPLA6,REEP1,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE26,ZFYVE27
GO:CC	axon cytoplasm	GO:1904115	4.09E-05	KIF1A,KIF5A,SPAST,SPG7
GO:CC	neuron projection cytoplasm	GO:0120111	0.000142	KIF1A,KIF5A,SPAST,SPG7
GO:CC	neuron projection	GO:0043005	0.000287	ATL1,ATP13A2,CPT1C,KIF1A,KIF5A,MAG,SPAST,SPG11,SPG7,ZFYVE27
GO:CC	integral component of membrane	GO:0016021	0.000445	ATL1,ATP13A2,BSCL2,C19ORF12,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIDINS220,MAG,NIPA1,PLP1,PNPLA6,REEP1,SLC16A2,SPAST,SPG7,ZFYVE27
GO:CC	intrinsic component of membrane	GO:0031224	0.000642	ATL1,ATP13A2,BSCL2,C19ORF12,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIDINS220,MAG,NIPA1,PLP1,PNPLA6,REEP1,SLC16A2,SPAST,SPG7,ZFYVE27
GO:CC	plasma membrane bounded cell projection	GO:0120025	0.002847	ATL1,ATP13A2,CPT1C,HSPD1,KIF1A,KIF5A,MAG,SPAST,SPG11,SPG7,ZFYVE27
GO:CC	plasma membrane bounded cell projection cytoplasm	GO:0032838	0.00326	KIF1A,KIF5A,SPAST,SPG7
GO:CC	cell projection	GO:0042995	0.003691	ATL1,ATP13A2,CPT1C,HSPD1,KIF1A,KIF5A,MAG,SPAST,SPG11,SPG7,ZFYVE27
GO:CC	cytoplasmic region	GO:0099568	0.005293	KIF1A,KIF5A,SPAST,SPG7
GO:CC	somatodendritic compartment	GO:0036477	0.010352	ATP13A2,CPT1C,KIF1A,KIF5A,SPG11,ZFYVE27
GO:CC	dendrite	GO:0030425	0.012927	CPT1C,KIF1A,KIF5A,SPG11,ZFYVE27
GO:CC	dendritic tree	GO:0097447	0.012927	CPT1C,KIF1A,KIF5A,SPG11,ZFYVE27
GO:CC	m-AAA complex	GO:0005745	0.014502	SPG7
GO:CC	endoplasmic reticulum tubular network membrane	GO:0098826	0.029626	ATL1
GO:CC	membrane protein complex	GO:0098796	0.049662	AP4S1,CPT1C,HSPD1,PLP1,SPG7,UBAP1

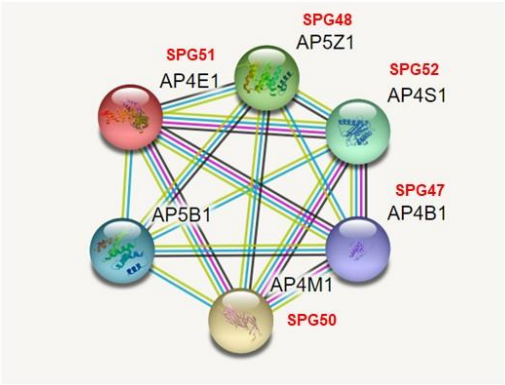
Gene Ontology enrichment analysis on proteins implicated in overlapping disorders				
source	**term_name**	**term_id**	**adjusted_**	**intersections**
GO:MF	ATP-dependent activity	GO:0140657	4.95E-05	ABCD1,AFG3L2,ATP1A1,ATP2B3,ATP7A,DYNC1H1,SETX
GO:MF	nucleoside-triphosphatase activity	GO:0017111	9.05E-05	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2
GO:MF	hydrolase activity, acting on acid anhydrides, in phosphorus-cont	GO:0016818	0.000108	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2
GO:MF	pyrophosphatase activity	GO:0016462	0.000108	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2
GO:MF	hydrolase activity, acting on acid anhydrides	GO:0016817	0.000108	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2
GO:MF	ATP hydrolysis activity	GO:0016887	0.00036	ABCD1,AFG3L2,ATP1A1,ATP2B3,ATP7A
GO:MF	purine ribonucleoside triphosphate binding	GO:0035639	0.000444	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	purine ribonucleotide binding	GO:0032555	0.000527	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	ribonucleotide binding	GO:0032553	0.000527	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	purine nucleotide binding	GO:0017076	0.000527	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	nucleoside phosphate binding	GO:1901265	0.001134	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	nucleotide binding	GO:0000166	0.001134	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	carbohydrate derivative binding	GO:0097367	0.001475	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	anion binding	GO:0043168	0.002	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	hydrolase activity	GO:0016787	0.00215	ABCD1,ABHD12,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,SETX
GO:MF	small molecule binding	GO:0036094	0.00251	ABCD1,AFG3L2,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SETX
GO:MF	ATP binding	GO:0005524	0.002875	ABCD1,AFG3L2,ATP1A1,ATP2B3,ATP7A,DYNC1H1,SETX
GO:MF	adenyl ribonucleotide binding	GO:0032559	0.003574	ABCD1,AFG3L2,ATP1A1,ATP2B3,ATP7A,DYNC1H1,SETX
GO:MF	adenyl nucleotide binding	GO:0030554	0.003613	ABCD1,AFG3L2,ATP1A1,ATP2B3,ATP7A,DYNC1H1,SETX
GO:MF	ATP-dependent peptidase activity	GO:0004176	0.024325	AFG3L2
GO:BP	establishment of localization	GO:0051234	0.002316	ABCD1,AFG3L2,ALS2,ANO10,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SCN11A,SPTBN2,VAMP1
GO:BP	plasma membrane bounded cell projection morphogenesis	GO:0120039	0.002316	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL
GO:BP	regulation of cellular component size	GO:0032535	0.002316	ALS2,ATP7A,DNM2,NEFL,SPTBN2
GO:BP	neuron projection development	GO:0031175	0.002316	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	transport	GO:0006810	0.002316	ABCD1,AFG3L2,ALS2,ANO10,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SCN11A,SPTBN2,VAMP1
GO:BP	cell projection morphogenesis	GO:0048858	0.002316	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL
GO:BP	neuron projection morphogenesis	GO:0048812	0.002316	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL
GO:BP	cell part morphogenesis	GO:0032990	0.00234	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL
GO:BP	cell projection organization	GO:0030030	0.002853	ABCD1,AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	axonogenesis	GO:0007409	0.002853	AFG3L2,ALS2,DNM2,NEFH,NEFL
GO:BP	neuron development	GO:0048666	0.002853	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	plasma membrane bounded cell projection organization	GO:0120036	0.002853	ABCD1,AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	localization	GO:0051179	0.002935	ABCD1,AFG3L2,ALS2,ANO10,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SCN11A,SPTBN2,SYNE1,VAMP1
GO:BP	cellular component morphogenesis	GO:0032989	0.002935	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL
GO:BP	organelle transport along microtubule	GO:0072384	0.003256	DYNC1H1,NEFH,NEFL
GO:BP	axon development	GO:0061564	0.003331	AFG3L2,ALS2,DNM2,NEFH,NEFL
GO:BP	regulation of anatomical structure size	GO:0090066	0.003446	ALS2,ATP7A,DNM2,NEFL,SPTBN2
GO:BP	endomembrane system organization	GO:0010256	0.005188	ABCD1,ALS2,ATL3,DNM2,SYNE1
GO:BP	cell morphogenesis involved in neuron differentiation	GO:0048667	0.005669	AFG3L2,ALS2,DNM2,NEFH,NEFL
GO:BP	neuron differentiation	GO:0030182	0.006126	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	nervous system development	GO:0007399	0.006126	ABCD1,AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX,SPTBN2
GO:BP	retrograde axonal transport	GO:0008090	0.006126	DYNC1H1,NEFL
GO:BP	regulation of biological quality	GO:0065008	0.006445	ABCD1,AFG3L2,ALS2,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFL,SCN11A,SPTBN2
GO:BP	generation of neurons	GO:0048699	0.007274	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	cell morphogenesis	GO:0000902	0.007745	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL
GO:BP	transport along microtubule	GO:0010970	0.010355	DYNC1H1,NEFH,NEFL
GO:BP	cell morphogenesis involved in differentiation	GO:0000904	0.01112	AFG3L2,ALS2,DNM2,NEFH,NEFL
GO:BP	neurogenesis	GO:0022008	0.011875	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	establishment of organelle localization	GO:0051656	0.011875	DNM2,DYNC1H1,NEFH,NEFL
GO:BP	cytoskeleton-dependent intracellular transport	GO:0030705	0.014586	DYNC1H1,NEFH,NEFL
GO:BP	microtubule-based transport	GO:0099111	0.01463	DYNC1H1,NEFH,NEFL
GO:BP	organelle localization	GO:0051640	0.02201	DNM2,DYNC1H1,NEFH,NEFL
GO:BP	axonal transport	GO:0098930	0.023292	DYNC1H1,NEFL
GO:BP	cell development	GO:0048468	0.027244	AFG3L2,ALS2,ATP7A,DNM2,NEFH,NEFL,SETX
GO:BP	axo-dendritic transport	GO:0008088	0.027254	DYNC1H1,NEFL
GO:BP	cellular localization	GO:0051641	0.038191	ABCD1,ALS2,DNM2,DYNC1H1,NEFH,NEFL,SPTBN2,SYNE1
GO:BP	microtubule-based movement	GO:0007018	0.044765	DYNC1H1,NEFH,NEFL
GO:BP	membrane organization	GO:0061024	0.0497	ABCD1,AFG3L2,DNM2,VAMP1
GO:CC	axon	GO:0030424	4.66E-10	ALS2,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SACS,SCN11A,SETX
GO:CC	neuron projection	GO:0043005	1.36E-09	ABHD12,ALS2,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SACS,SCN11A,SETX,VAMP1
GO:CC	cell projection	GO:0042995	4.65E-08	ABHD12,ALS2,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SACS,SCN11A,SETX,SPTBN2,VAMP1
GO:CC	plasma membrane bounded cell projection	GO:0120025	3.32E-07	ABHD12,ALS2,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,NEFH,NEFL,SACS,SCN11A,SETX,VAMP1
GO:CC	neuron projection cytoplasm	GO:0120111	0.001131	ABHD12,DYNC1H1,NEFL
GO:CC	somatodendritic compartment	GO:0036477	0.009977	ABHD12,ALS2,ATP7A,SACS,SPTBN2
GO:CC	plasma membrane bounded cell projection cytoplasm	GO:0032838	0.010268	ABHD12,DYNC1H1,NEFL
GO:CC	membrane protein complex	GO:0098796	0.010268	ABHD12,AFG3L2,ATP1A1,SCN11A,SYNE1,VAMP1
GO:CC	axon cytoplasm	GO:1904115	0.014002	DYNC1H1,NEFL
GO:CC	m-AAA complex	GO:0005745	0.015112	AFG3L2
GO:CC	cytoplasmic region	GO:0099568	0.015112	ABHD12,DYNC1H1,NEFL
GO:CC	dendrite	GO:0030425	0.016183	ABHD12,ALS2,ATP7A,SACS
GO:CC	dendritic tree	GO:0097447	0.016183	ABHD12,ALS2,ATP7A,SACS
GO:CC	organelle membrane	GO:0031090	0.021503	ABCD1,ABHD12,AFG3L2,ATL3,ATP1A1,ATP7A,DNM2,SYNE1,VAMP1
GO:CC	endoplasmic reticulum tubular network membrane	GO:0098826	0.02582	ATL3
GO:CC	endomembrane system	GO:0012505	0.0261	ABCD1,ABHD12,ALS2,ATL3,ATP1A1,ATP7A,DNM2,DYNC1H1,SYNE1,VAMP1
GO:CC	integral component of membrane	GO:0016021	0.030373	ABCD1,ABHD12,AFG3L2,ANO10,ATL3,ATP1A1,ATP2B3,ATP7A,SCN11A,SYNE1,VAMP1
GO:CC	membrane	GO:0016020	0.031255	ABCD1,ABHD12,AFG3L2,ALS2,ANO10,ATL3,ATP1A1,ATP2B3,ATP7A,DNM2,DYNC1H1,SCN11A,SPTBN2,SYNE1,VAMP1
GO:CC	cytoplasm	GO:0005737	0.031255	ABCD1,ABHD12,AFG3L2,ALS2,ATL3,ATP1A1,ATP7A,CAMTA1,DNM2,DYNC1H1,NEFH,NEFL,SACS,SETX,SPTBN2,SYNE1,VAMP1
GO:CC	intrinsic component of membrane	GO:0031224	0.03333	ABCD1,ABHD12,AFG3L2,ANO10,ATL3,ATP1A1,ATP2B3,ATP7A,SCN11A,SYNE1,VAMP1
GO:CC	organelle subcompartment	GO:0031984	0.036528	ABCD1,ABHD12,ATL3,ATP7A,DNM2

Gene Ontology enrichment analysis on known HSP proteins			
source	**term_name**	**term_id**	**intersections**
GO:BP	establishment of localization	GO:0051234	AP4S1,ATP13A2,CAPN1,HSPD1,KIF1A,KIF5A,NIPA1,PLP1,REEP1,SLC16A2,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE27
GO:BP	transport	GO:0006810	AP4S1,ATP13A2,CAPN1,HSPD1,KIF1A,KIF5A,NIPA1,PLP1,SLC16A2,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE27
GO:BP	localization	GO:0051179	AP4S1,ATP13A2,BSCL2,CAPN1,CYP7B1,HSPD1,KIF1A,KIF5A,NIPA1,PLP1,REEP1,SLC16A2,SPAST,SPG11,SPG7,TECPR2,UBAP1,WASHC5,ZFYVE27
GO:CC	organelle membrane	GO:0031090	ALDH18A1,AP4S1,ATL1,ATP13A2,BSCL2,C19ORF12,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIF1A,PNPLA6,REEP1,SPAST,SPG11,SPG7,UBAP1,ZFYVE26,ZFYVE27
GO:CC	membrane	GO:0016020	ALDH18A1,AP4S1,ATL1,ATP13A2,BSCL2,C19ORF12,CAPN1,CPT1C,CYP7B1,ERLIN2,FA2H,GBA2,HSPD1,KIDINS220,KIF1A,KIF5A,MAG,MARS1,NIPA1,PLP1,PNPLA6,REEP1,SLC16A2,SPAST,SPG11,SPG7,UBAP1,ZFYVE26,ZFYVE27
GO:CC	plasma membrane bounded cell projection	GO:0120025	ATL1,ATP13A2,CPT1C,HSPD1,KIF1A,KIF5A,MAG,SPAST,SPG11,SPG7,ZFYVE27
GO:CC	cell projection	GO:0042995	ATL1,ATP13A2,CPT1C,HSPD1,KIF1A,KIF5A,MAG,SPAST,SPG11,SPG7,ZFYVE27
GO:CC	vesicle	GO:0031982	AP4S1,ATP13A2,CAPN1,HSPD1,KIDINS220,KIF1A,MARS1,NIPA1,SPAST,SPG11,UBAP1,WASHC5,ZFYVE26,ZFYVE27
Gene Ontology enrichment analysis on proteins involved in mimicking disorders			
source	**term_name**	**term_id**	**intersections**
GO:BP	localization	GO:0051179	AAAS,CACNA1E,CSF1R,CTNNB1,DNM1L,KCNA2,LYST,MCOLN1,PDGFB,PLA2G6,SLC20A2,SLC2A1,SLC6A19,TINF2
GO:BP	transport	GO:0006810	AAAS,CACNA1E,CTNNB1,DNM1L,KCNA2,LYST,MCOLN1,PDGFB,PLA2G6,SLC20A2,SLC2A1,SLC6A19
GO:BP	establishment of localization	GO:0051234	AAAS,CACNA1E,CTNNB1,DNM1L,KCNA2,LYST,MCOLN1,PDGFB,PLA2G6,SLC20A2,SLC2A1,SLC6A19
GO:CC	membrane	GO:0016020	AAAS,CACNA1E,COL4A2,COL6A1,CSF1R,CTNNB1,DNM1L,GCH1,KCNA2,LYST,MCOLN1,PDGFB,PLA2G6,POLR3A,SLC20A2,SLC2A1,SLC6A19
GO:CC	vesicle	GO:0031982	COL4A2,COL6A1,CTNNB1,DNM1L,GCH1,MCOLN1,PDGFB,SLC20A2,SLC2A1,SLC6A19,TUBB4A
GO:CC	cell projection	GO:0042995	CTNNB1,GCH1,KCNA2,MCOLN1,PLA2G6,SLC6A19,TUBB4A
GO:CC	plasma membrane bounded cell projection	GO:0120025	CTNNB1,GCH1,KCNA2,PLA2G6,SLC6A19,TUBB4A
GO:CC	organelle membrane	GO:0031090	AAAS,COL6A1,DNM1L,GCH1,KCNA2,MCOLN1,PDGFB,SLC2A1

Supplementary Table 2.5. Detected copy number variations using genome sequencing.

ID	Gene	ID	Type
FSP-06-017	DNM1L	chr12:32713010-32718442	Deletion
FSP-04-070, FSP-01-198	DNM1L	chr12:32716228-32718978; chr12:32716228-32719996	Deletion
FSP-01-081	TBL1X	chrX:9738917-9739949	Duplication
FSP-06-002	OPHN1	chrX:68332049-68591726	Duplication
FSP-06-019	DCC	chr18:53205165-53207276	Deletion





Supplementary Figure 2.1. Protein-protein interaction between the known and potential new HSP genes.

- **Chapter 3**

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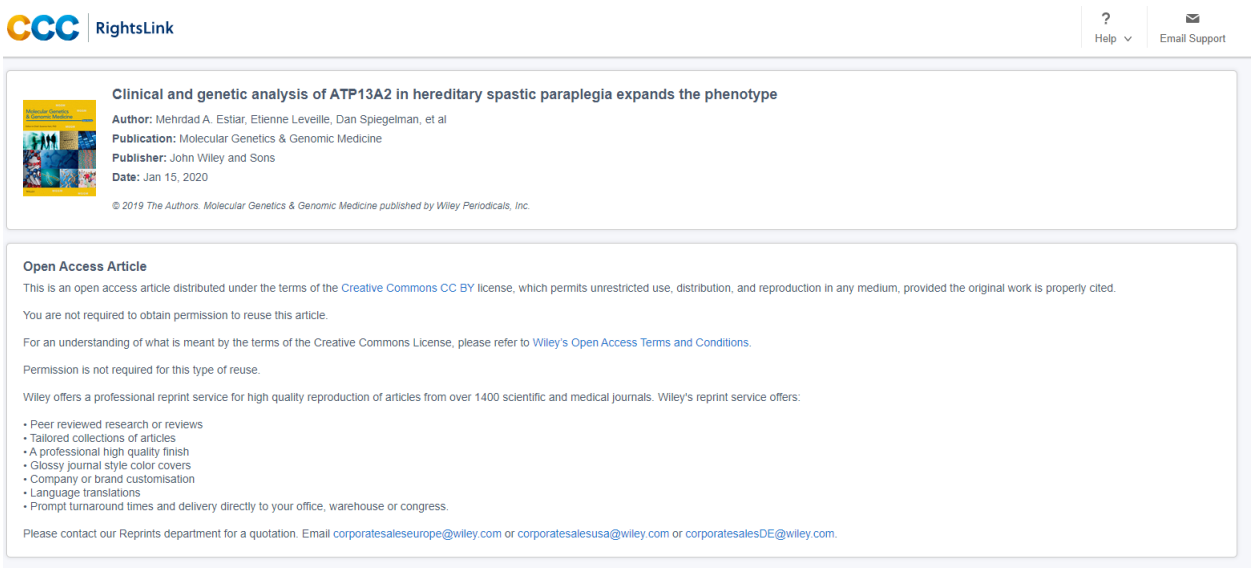

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Clinical and genetic analysis of ATP13A2 in hereditary spastic paraplegia expands the phenotype

Author: Mehrdad A. Estiar, Etienne Leveille, Dan Spiegelman, et al

Publication: Molecular Genetics & Genomic Medicine

Publisher: John Wiley and Sons

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Supplementary Table 1. Pathway enrichment analysis of genes which are known to be involved in HSP, ALS and Parkinsonism disorders. The common pathways in three mentioned diseases are highlighted in green.

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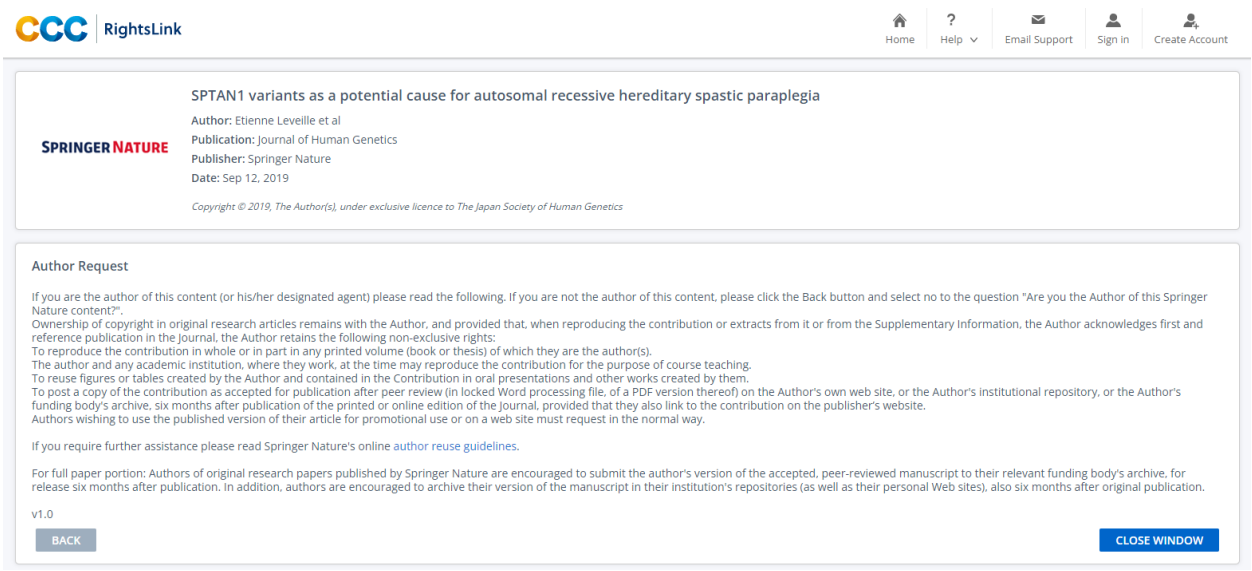

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SPTAN1 variants as a potential cause for autosomal recessive hereditary spastic paraplegia

Author: Etienne Leveille et al
Publication: Journal of Human Genetics
Publisher: Springer Nature
Date: Sep 12, 2019

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- Ziv Gan-Or, Dr. <ziv.gan-or@mcgill.ca>

Hi Mehrdad,

Great to hear from you, I am doing well and I hope you are too. Congratulations on getting close to graduation! I have no issues with you using the *SPTAN1* paper. Good luck with your thesis.

Best,

Etienne

From: Mehrdad Asghari Estiar <mehrdad.a.estiar@mail.mcgill.ca>
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Hi Etienne,

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Supplementary Table 1. genes that are known or suspected to be involved in HSP or other similar disorders

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https://www.nature.com/articles/s10038-019-0669-2

Supplementary Table 2. The results of SPTAN1 pathway analysis using WebGestalt.

Pathway	HSP genes	P value	FDR
Intracellular transport	*TFG, AP4B1, VPS37A, ARL6IP1, DDHD2, ATP13A2, AP4E1, HSPD1, KIF5A, REEP2, KIF1A, RTN2, REEP1, SPAST, UCHL1, SPG11, AP4M1*	1.30E-05	2.48E-02
Axon development	*ZFYVE27, SPG20, KIF5A, L1CAM, MAG, ATL1, PLP1, UCHL1, SPG11*	1.89E-04	3.60E-02
Axon	*ZFYVE27, CPT1C, L1CAM, MAG, ATL1, KIF1A, SPAST, UCHL1, SPG11*	9.56E-06	1.23E-03

- **Chapter 5**

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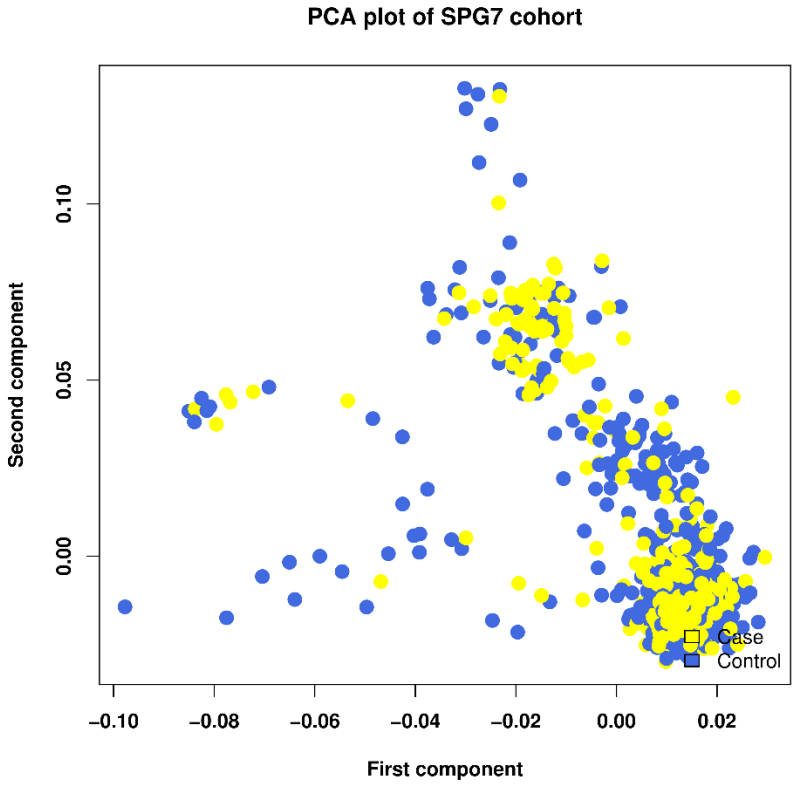
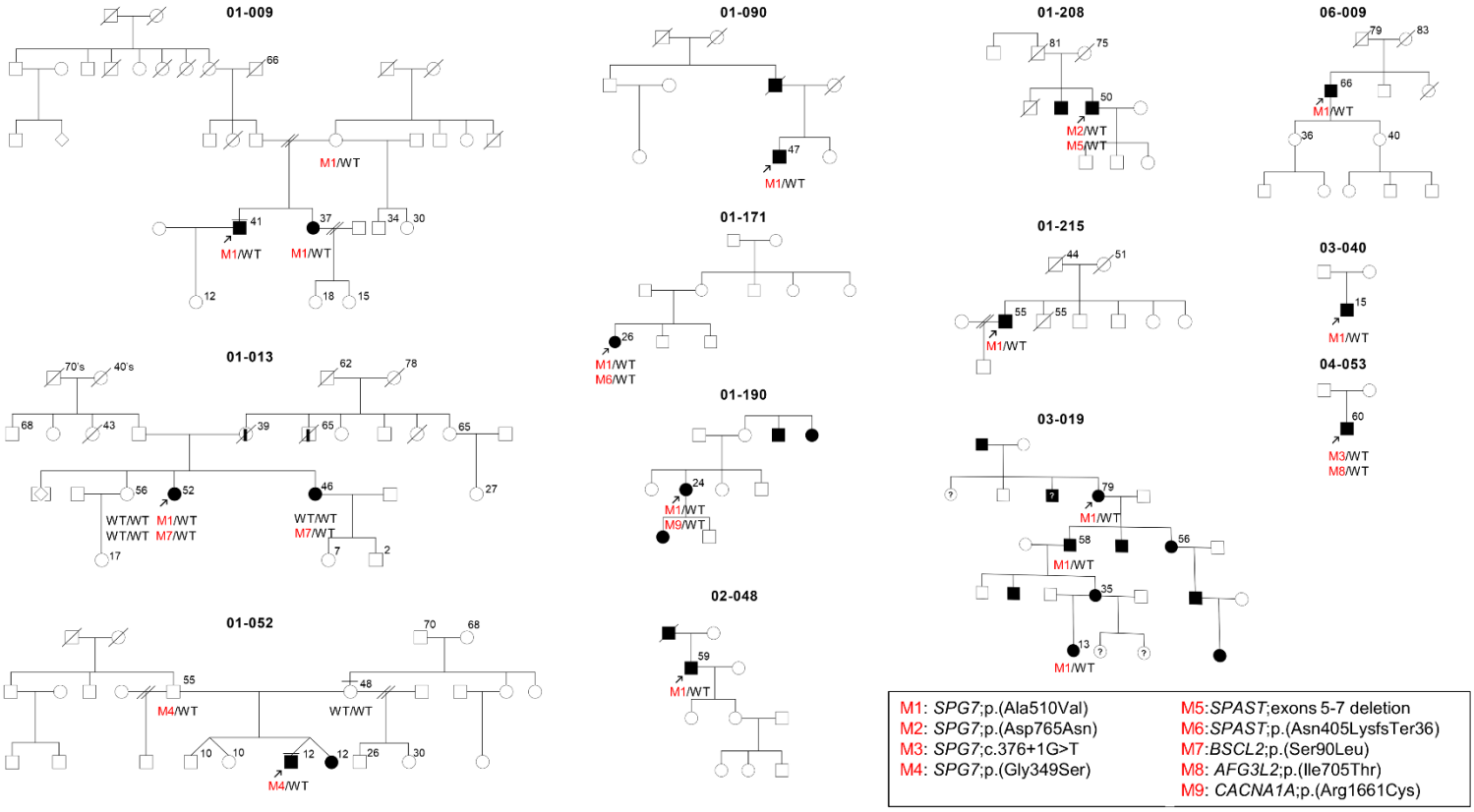


Supplementary Figure 1. Principal component analysis (PCA) on common variants was used to test for the presence of population stratification.



Supplementary Figure 2. Pedigrees of families with heterozygous *SPG7* variants with and without additional variants in other hereditary spastic paraplegia (HSP)-related or spastic paraplegia 7 (*SPG7*)-related genes.

Supplementary Table 1. Genetic and clinical data of hereditary spastic paraplegia (HSP) subjects included in this study.

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Supplementary Table 2. Characteristics of control individuals.

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Supplementary Table 3. A complete list of genes associated with spastic paraplegia phenotype. The list includes known hereditary spastic paraplegia (HSP) genes (https://omim.org/phenotypicSeries/PS303350), the list of in-house genes, gene panel for movement disorders (https://www.radboudumc.nl/en/patientenzorg/onderzoeken/exome-sequencing-diagnostics/exomepanelspreviousversions/exoompanels-huidige-en-voorgaande-versies/movement-disorders) and genes from HSPome.

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Supplementary Table 4. List of *SPG7* variants which were detected in all the subjects included in this study.

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Supplementary Table 5. Gene Ontology (GO) term enrichment with respect to Biological Process (BP) using g:Profiler. *P* values were corrected for multiple testing using the Benjamini−Hochberg false discovery rate. Adjusted P values show the significance of enrichment with the threshold of $P < 0.05$. The data source from GO with BP subontology was used to describe in which biological process the gene product participates.

source	term_name	term_id	adjusted_	negative_	term_size	query_siz	intersecti	effective_	intersections
GO:BP	axonal transport	GO:0098930	0.006708	2.173433	59	4	2	17916	SPG7,SPAST
GO:BP	anterograde axonal transport	GO:0008089	0.006708	2.173433	49	4	2	17916	SPG7,SPAST
GO:BP	axo-dendritic transport	GO:0008088	0.006708	2.173433	70	4	2	17916	SPG7,SPAST
GO:BP	transport along microtubule	GO:0010970	0.012139	1.915817	163	4	2	17916	SPG7,SPAST
GO:BP	microtubule-based process	GO:0007017	0.012139	1.915817	784	4	3	17916	SPG7,SPAST,TBCE
GO:BP	microtubule-based transport	GO:0099111	0.013906	1.856786	193	4	2	17916	SPG7,SPAST
GO:BP	cytoskeleton-dependent intracellular transport	GO:0030705	0.014096	1.850911	203	4	2	17916	SPG7,SPAST
GO:BP	microtubule-based movement	GO:0007018	0.023369	1.631351	293	4	2	17916	SPG7,SPAST
GO:BP	organelle organization	GO:0006996	0.026174	1.582123	3988	4	4	17916	SPG7,SPAST,BSCL2,TBCE
GO:CC	neuron projection cytoplasm	GO:0120111	0.00351	2.454675	87	4	2	18856	SPG7,SPAST
GO:CC	axon cytoplasm	GO:1904115	0.00351	2.454675	58	4	2	18856	SPG7,SPAST
GO:CC	m-AAA complex	GO:0005745	0.008697	2.06064	2	4	1	18856	SPG7
GO:CC	plasma membrane bounded cell projection cytoplasm	GO:0032838	0.011594	1.935771	209	4	2	18856	SPG7,SPAST
GO:CC	mitochondrial permeability transition pore complex	GO:0005757	0.011594	1.935771	4	4	1	18856	SPG7
GO:CC	cytoplasmic region	GO:0099568	0.012284	1.910643	252	4	2	18856	SPG7,SPAST
GO:CC	integral component of organelle membrane	GO:0031301	0.023007	1.63814	370	4	2	18856	SPG7,BSCL2
GO:CC	intrinsic component of organelle membrane	GO:0031300	0.023973	1.62028	401	4	2	18856	SPG7,BSCL2
GO:CC	axon	GO:0030424	0.046867	1.329129	653	4	2	18856	SPG7,SPAST

Supplementary Table 6. List of spastic paraplegia 7 (SPG7)-interacting genes.

Source	Gene Symbol
g:Profiler	BAX
g:Profiler	PPIF
g:Profiler	SPG7
g:Profiler	VDAC1
g:Profiler	AFG3L2
STRING	MCU
STRING	CCDC109B
STRING	PPIF
STRING	VDAC1
STRING	PHB2
STRING	PHB
STRING	PMPCB
STRING	PMPCA
STRING	C2orf47
GeneMANIA	YME1L1
GeneMANIA	ELF3
GeneMANIA	CERK
GeneMANIA	GPR55
GeneMANIA	SPATS1
GeneMANIA	SRSF3
GeneMANIA	HNRNPR
GeneMANIA	NDC80
GeneMANIA	NDUFB9
GeneMANIA	SLC25A3
GeneMANIA	SFXN2
GeneMANIA	KIF7
GeneMANIA	PDK1
GeneMANIA	RING1
GeneMANIA	MORC2
GeneMANIA	PLXNB1
GeneMANIA	ZNF512B
GeneMANIA	ULK3
GeneCards	AP-1
GeneCards	ATF2
GeneCards	Jun
GeneCards	CEBPb
GeneCards	NFKB1
TheSignalingPathwayProject	CDK4

TheSignalingPathwayProject	NR2F1
TheSignalingPathwayProject	EP300
TheSignalingPathwayProject	MXI1
TheSignalingPathwayProject	MAX
TheSignalingPathwayProject	ATRA
TheSignalingPathwayProject	FOSL2
TheSignalingPathwayProject	CTCF
TheSignalingPathwayProject	REST
TheSignalingPathwayProject	FOXP2
TheSignalingPathwayProject	GABPA
TheSignalingPathwayProject	BRD4
TheSignalingPathwayProject	JQ1
TheSignalingPathwayProject	TAF1
TheSignalingPathwayProject	MTUS2
TheSignalingPathwayProject	PNMA1
TheSignalingPathwayProject	AKT1
TheSignalingPathwayProject	AP3S1
HP	ATP13A2
HP	AMPD2
HP	AP4B1
HP	DSTYK
HP	GJC2
HP	IBA57
HP	SELENOI
HP	KIF1A
HP	TFG
HP	UCHL1
HP	CYP2U1
HP	FARS2
HP	AP5Z1
HP	AP4M1
HP	VPS37A
HP	ERLIN2
HP	DDHD2
HP	CYP7B1
HP	GBA2
HP	SPG27
HP	ALDH18A1
HP	ENTPD1
HP	ERLIN1
HP	NT5C2

HP	CAPN1
HP	B4GALNT1
HP	C12orf65
HP	SPG20
HP	SPG24
HP	SPG32
HP	AP4S1
HP	DDHD1
HP	ZFYVE26
HP	TECPR2
HP	SPG11
HP	AP4E1
HP	ACP33
HP	ARL6IP1
HP	FA2H
HP	PGN
HP	PCYT2
HP	PNPLA6
HP	C19orf12
HP	MAG
HP	SPG16
HP	PLP1
HP	SPG34
HP	L1CAM
HP	EPT1
Klutho, Paula J., et al. "Genetic manipulation of SPG7 or NipSnap2 does not affect mitochondrial permeability transition." Cell Death Discovery 6.1 (2020): 1-3.	NIPSNAP1
Klutho, Paula J., et al. "Genetic manipulation of SPG7 or NipSnap2 does not affect mitochondrial permeability transition." Cell Death Discovery 6.1 (2020): 1-3.	NIPSNAP2
Klutho, Paula J., et al. "Genetic manipulation of SPG7 or NipSnap2 does not affect mitochondrial permeability transition." Cell Death Discovery 6.1 (2020): 1-3.	CypD
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Supplementary Table 7. Gene Ontology (GO) term enrichment using g:Profiler (Benjamini−Hochberg adjusted P values set at <0.05).

It can be accessible:

https://movementdisorders.onlinelibrary.wiley.com/doi/10.1002/mds.28528

Supplementary Table 8. (a) Comparison of clinical characteristics in different groups based on variant type. (b) Comparison of clinical characteristics in different groups based on p.(Ala510Val) variant.

Supplementary Table 8a.

Variable	Two missense	Two LoF	One missense & one LoF	One missense	One LoF	*P* value
Age at onset ± SD years	32.6 ± 16.8 (n=15)	33.2 ± 16.3 (n=8)	28.5 ± 12.2 (n=7)	20.6 ± 20.8 (n=12)	-	0.271
Spastic Paraplegia Rating Scale (SPRS) score	16.0 ± 6.7 (n=12)	16.8 ± 8.3 (n=5)	16.6 ± 6.2 (n=5)	30.0 ± 7.4 (n=4)	9 (n=1)	0.083
Gender (M/F)	8/9	4/2	8/0	4/7	1/0	0.047
Lower extremity weakness	12/14	6/8	4/7	9/12	0/1	0.301
Lower extremity spasticity	14/14	8/8	6/7	11/12	1/1	0.577
Lower extremity hyperreflexia	13/14	8/8	7/7	11/12	0/1	0.007
Extensor plantar responses	12/14	7/8	6/7	11/11	0/1	0.066
Abnormal bladder function	6/14	3/8	2/7	8/11	0/1	0.272
Ankle clonus	10/14	7/8	5/6	4/10	1/1	0.178
Motor delay	0/14	0/8	0/7	2/8	0/1	0.095
Learning disability	1/14	0/8	0/7	0/7	0/1	0.793
Progressive cognitive deficits	1/14	1/8	0/7	1/9	0/1	0.897
Retinopathy or optic atrophy	0/13	0/8	1/7	0/9	0/1	0.337
Ocular movement abnormalities	4/14	2/8	1/7	1/9	0/1	0.814
Deafness	1/13	0/8	0/7	0/9	0/1	0.740
Swallowing difficulties	1/13	1/8	0/7	1/9	0/1	0.902
Dysarthria	6/14	2/8	4/6	1/9	0/1	0.183
Upper extremity weakness	2/13	1/8	0/7	0/9	0/1	0.610
Upper extremity hyperreflexia	5/14	2/8	1/7	2/9	1/1	0.433
Amyotrophy or lower motor neuron features	1/13	2/8	1/6	1/9	0/1	0.821
Sensory abnormalities	5/14	2/8	2/7	2/9	0/1	0.912
Peripheral neuropathy	2/12	0/7	3/7	3/8	0/1	0.268
Pes cavus	4/13	1/8	1/7	4/9	1/1	0.257
Ataxic gait	7/13	2/8	4/7	1/9	0/1	0.166
Upper extremity ataxia	5/14	3/8	3/6	0/9	0/1	0.202
Upper extremity intent tremor	4/14	3/8	2/7	0/9	0/1	0.368
Lower extremity ataxia	7/14	2/8	4/6	1/9	0/1	0.138

Lower extremity intent tremor	5/13	2/8	3/7	1/9	0/1	0.543
Seizures	1/13	0/8	0/7	0/9	0/1	0.740
Skeletal abnormalities	0/13	0/8	0/7	1/9	0/1	0.507
Myoclonus	0/13	0/8	0/6	1/9	0/1	0.525
Abnormal brain MRI	3/10	1/5	3/6	1/7	0	0.524
Abnormal spine MRI	0/10	0/4	0/6	1/7	0	0.397

SD = Standard Deviation; LoF = Loss of Function; MRI = magnetic resonance imaging.

The corrected *p*-value threshold was $p < 0.001$. None of the clinical features significance passes Bonferroni correction.

Supplementary Table 8b.

Variable	Het A510V	Comp Het A510V	Homo A510V	Others	*P* value
Age at onset ± SD	18.50 ± 20.27 (n=6)	31.42 ± 15.70 (n=12)	37.14 ± 18.40 (n=7)	25.28 ± 17.64 (n=18)	0.165
Spastic Paraplegia Rating Scale (SPRS) score	29.33 ± 9.01 (n=3)	17.00 ± 7.87 (n=7)	15.33 ± 6.77 (n=6)	17.18 ± 7.88 (n=11)	0.237
Lower extremity weakness	6/6	9/12	6/7	10/18	0.141
Lower extremity spasticity	6/6	11/12	7/7	16/18	0.686
Lower extremity hyperreflexia	6/6	12/12	7/7	14/18	0.106
Extensor plantar responses	6/6	8/11	6/7	16/18	0.447
Abnormal bladder function	6/6	4/12	2/7	7/17	**0.031**
Ankle clonus	4/6	7/10	5/7	11/17	0.987
Motor delay	2/4	0/12	0/7	0/16	0.086
Learning disability	0/3	1/12	0/7	0/16	0.527
Progressive cognitive deficits	0/6	1/12	1/7	1/15	0.807
Retinopathy or optic atrophy	0/6	1/11	0/7	0/15	0.455
Ocular movement abnormalities	0/6	3/12	0/7	5/15	0.164
Deafness	0/6	0/11	1/7	0/15	0.196
Swallowing difficulties	0/6	2/11	0/7	1/15	0.422
Dysarthria	0/6	6/11	2/7	5/15	0.151
Upper extremity weakness	0/6	2/11	1/7	0/15	0.274
Upper extremity hyperreflexia	0/6	5/12	3/7	3/15	0.192
Amyotrophy or lower motor neuron features	0/6	3/10	0/7	2/15	0.217
Sensory abnormalities	1/5	4/12	3/7	3/16	0.617
Peripheral neuropathy	2/5	3/10	1/6	2/15	0.561
Pes cavus	2/6	4/11	3/7	2/15	0.420
Ataxic gait	0/6	5/11	3/7	6/15	0.256
Upper extremity ataxia	0/6	3/11	2/7	6/15	0.335
Upper extremity intent tremor	0/6	2/12	2/7	5/15	0.371
Lower extremity ataxia	0/6	6/11	2/7	6/15	0.151

Lower extremity intent tremor	0/6	4/11	3/7	4/15	0.324
Seizures	0/6	0/11	0/7	1/15	0.650
Skeletal abnormalities	0/6	1/11	0/7	0/15	0.455
Myoclonus	0/6	1/10	0/7	0/15	0.411
Abnormal brain MRI	0/3	3/10	2/4	3/11	0.548
Abnormal spine MRI	1/4	0/9	0/5	0/9	0.113

SD = Standard Deviation; Het = Heterozygous; Homo = Homozygous; Comp Het = Compound Heterozygous; MRI = magnetic resonance imaging.

The corrected *p*-value threshold was $p < 0.0005$. None of the clinical features significance passes Bonferroni correction.