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Clinical and genetic spectra of 1550 index patients with hereditary spastic paraplegia

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Abstract

Hereditary spastic paraplegia refers to rare genetic neurodevelopmental and/or neurodegenerative disorders in which spasticity due to length-dependent damage to the upper motor neuron is a core sign. Their high clinical and genetic heterogeneity makes their diagnosis challenging.

Multigene panels allow a high-throughput targeted analysis of the increasing number of genes involved using next-generation sequencing. We report here the clinical and genetic results of 1550 index cases tested for variants in a panel of hereditary spastic paraplegia related genes analyzed in routine diagnosis.

A causative variant was found in 475 patients (30.7%) in 35/65 screened genes. *SPAST* and *SPG7* were the most frequently mutated genes, representing 142 (9.2%) and 75 (4.8%) index cases of the whole series, respectively. *KIF1A*, *ATL1*, *SPG11*, *KIF5A* and *REEP1* represented more than 1% (> 17 cases) each. There were 661 causative variants (382 different ones) and 30 of them were structural variants. This large cohort allowed us obtaining an overview of the clinical and genetic spectrum of hereditary spastic paraplegia in clinical practice. Because of the wide phenotypic variability, there was no very specific sign that could predict the causative gene but there were some constellations of symptoms that were found often related to specific subtypes. Finally, we confirmed the diagnostic effectiveness of a targeted sequencing panel as a first-line genetic test in hereditary spastic paraplegia. This is a pertinent

strategy because of the relative frequency of several known genes (i.e.: *SPAST*, *KIF1A*) and it allows identifying variants in the rarest involved genes and to detect structural rearrangements via coverage analysis, which is less efficient in exome data sets. It is crucial because these structural variants represent a significant proportion of the pathogenic hereditary spastic paraplegia variants (~6% of patients), notably for *SPAST* and *REEPI*.

In a subset of 42 index cases negative for the targeted multigene panel, subsequent whole exome sequencing allowed to reach a theoretical diagnosis yield of ~50%. We then propose a two-step strategy combining the use of a panel of genes followed by whole exome sequencing in negative cases.

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Abbreviations: AD = autosomal dominant; AR = autosomal recessive; CNV = copy number variant; ENMG = electroneuromyography; HSP = hereditary spastic paraplegia; iqr = interquartile range; MLPA= Multiplex Ligation Probe Amplification; NGS = next-generation sequencing; PTC = premature truncation codon;

TRP = target resequencing multigene panels; XL = X-linked

Introduction

Hereditary spastic paraplegia (HSP) is the umbrella term for a large group of neurodevelopmental and/or neurodegenerative disorders with a prevalence of 1 to 10/100000 people.¹ The number of causative genes identified is continuing to increase (> 80).^{2,3} Pathogenic variants in these HSP-related genes mainly cause length-dependant degeneration of upper motor neurons. The historical classification is based on the mode of inheritance (autosomal dominant [AD], autosomal recessive [AR], mitochondrial and X-linked [XL]), and on the pure or complex nature of the clinical presentation according to the presence of other neurological or extra-neurological features.⁴ However, wide phenotypic and inheritance modalities exist, and overlapping phenotypes with other neurological disorders are frequent,^{2,5} with considerable consequences for medical practice.

Next-generation sequencing (NGS), which is now commonly used in genetic diagnosis, consists of either exome sequencing or target resequencing multigene panels (TRPs) for a similar consumable cost nowadays.⁶ This second approach enables quick and high-throughput targeted screening of focused series of genes. Since processing and interpretation of generated data are the main limits of NGS approaches, targeting of already incriminated genes is recommended to facilitate and accelerate the analysis, and also avoid incidental findings according to ethical rules. Even if a targeted approach miss possible identification of novel causative genes, it is more suitable to detect gene rearrangements, which are relatively frequent in HSP. We report here the clinical and genetic results of 1550 index cases tested with an HSP-targeted multigene resequencing panel, designed in our centre and used in real-life routine diagnosis.

Materials and methods

Patients

We reviewed the results from 1550 HSP index cases tested between December 2013 and July 2019 with an HSP-related genes panel designed according to Morais et al.⁷ Patients were referred to the Neurogenetics laboratory of the Pitié-Salpêtrière University Hospital (n = 1282) or recruited through the French members of the SPATAX network (<https://spatax.wordpress.com/>) at the Centre for Biological Resources of the Paris Brain Institute - ICM (n = 268). These cases were distributed into two groups depending on whether the panel was used as a first genetic test (“naïve” cases) or as a second-line test after targeted genetic testing of the most frequent HSP genes according to the clinical presentation of the patients (mostly *SPAST*, *ATL1* or *SPG11*). All patients gave their written consent for genetic testing for diagnostic purposes, in accordance with French national ethical rules and with local French regulations for research (Paris Necker ethics committee approval [RBM 01-29 and RBM 03-48] to AB and AD). Clinical and genetic data were extracted from the SPATAX and Neurogenetics laboratory databases (Agreement from the Commission Informatique et Libertés), including the latest clinical follow-up and panel results. Functional disability was measured in terms of the SPATAX Functional Score ranging from 0 (no functional handicap) to 7 (confined to bed); the scale and clinical chart are available at: <https://spatax.files.wordpress.com/2013/09/fichecliniquespatax-eurospa-2011.pdf>.

Panel of spastic paraplegia genes

A custom NGS panel was developed at ICM, to search for rare non-silent variants in HSP-related genes. The procedures for the NGS panel, variant validation by Sanger sequencing or multiplex ligation-dependent probe amplification (MLPA, MRC Holland, The Netherlands) have previously been described.⁷ The core panel included 65 HSP-related genes (see list in Supplementary Table 1). In addition, seven genes were added to the panel for the last 373 patients (24.1%) (see list in Supplementary Table 1). An annotation and variant selection pipeline was developed by GenoDiag-GenoSplice, France. Structural variants were detected through comparative analysis of the read depth of all samples of a single run with an analysis using an in-house script. The detection was based on the coverage of each exon using BEDtools (v2-2.20.1). Normalization was applied in order to remove coverage bias according to the different samples using R (v3.3.2). The normalized coverage was then used to perform a z-score. Two additional parameters were computed: the coverage dispersion and the

coverage ratio, which measure the sequencing quality and the deletion or duplication significance. These three values were used to filter the false-positive results and set the events confidence level. For graphical output, the coverage plot by exon was performed using ggplot2 R library. Data interpretation was accepted on patient results fitting at least the following criteria: mean coverage of at least 30x and variant detected on at least 10 reads. Whenever possible, we checked family segregation using Sanger sequencing with BigDye chemistry on PCR amplicons (Applied Biosystems, USA) or MLPA. For a given patient, the panel result was considered positive if a pathogenic or likely pathogenic variant was identified. Pathogenic/likely pathogenic variants or variants of undetermined significance (VUS) were determined using the American College of Medical Genetics (ACMG) classification.⁸ When only one variant was identified in a phenotypically relevant gene associated with AR inheritance, we systematically searched for a second variant in regions not covered by Sanger sequencing and/or MLPA.

Exome Sequencing

Whole exome sequencing was performed in a subset of 42 index cases in whom no causative variant or copy number variation (CNV) could be identified using the panel of HSP genes. Standard procedures were used as reported elsewhere,⁹ focusing the analysis to the current list of 4493 genes with phenotype-causing mutation (<https://www.omim.org/statistics/geneMap>).

Statistical analysis

Categorical variables were described as numbers (percentages) and continuous variables as mean (with standard deviation) or median (with interquartile range [iqr]) according to their distribution. The statistical analyses were performed using R studio 1.3.1073 (<http://www.rstudio.com/>) with R 4.0.2. Chi-square or Fisher's exact test was used to compare qualitative variables and the Welch test to compare continuous variables, such as the medians of onset age and functional disability. A p-value < 0.05 was considered as statistically significant. Two-tailed hypotheses were considered throughout.

Data availability

The data supporting the findings of this study are available from the corresponding author, upon reasonable request, subject to institutional agreements and ethical approvals. The variants classified as pathogenic or of undetermined significance were shared in ClinVar

(entries SCV001450926 to SCV001451350, SCV001763703 to SCV001763706 and SCV001481805).

Results

Global genetic and clinical characteristics of the cohort

As summarized in Table 1, a majority of the 1550 index patients (840; 54.2%) were isolated cases (i.e., with no known family history of the disease) followed by AD (father-son transmission present) or probable AD/dominant (340; 21.9%) and AR forms (at least two affected sibs from unaffected parents or an isolated case with related parents) (154; 9.9%). X-linked inheritance (XL) was suspected in two males (0.1%), and inheritance was unknown for 214 patients (13.8%). Typically, inheritance was classified as unknown when either the information was unavailable or when the presence of multiple affected cases in a pedigree did not fit with a classical mode of transmission. Males ($n = 854$) were more frequently observed than females ($n = 688$) (55.1% vs 44.4%, $p = 0.003$). The median age at onset was 25.0 (iqr 40.8) years and the median disease duration was 12.4 (iqr 20.8) years (data available for 1402 patients) at a median age at last examination of 46.4 (iqr 31.0) years. The pure HSP forms were less frequent than complicated forms (43.9% vs 56.1%, $p < 0.001$). Patients mainly originated from Western Europe and Mediterranean countries.

The cohort comprised 809 (52.2%) patients for whom the panel was the first-line analysis (first-line group) and 741 patients (47.8%) for whom the analysis of the gene panel was performed after targeted genetic testing of the most frequent HSP genes or genes selected on the basis of the clinical presentation (second-line group) (Table 1). Some clinical characteristics differed between the two groups. A later age at onset was observed in the first-line compared to the second-line group (median of 32 versus 20 years, $p < 0.001$). This may be explained by the presence of more isolated cases in the first-line group (63.9% vs 43.6%, $p < 0.001$), with a later onset compared to the rest of the series (29.0 vs 25.0 years, $p = 0.01$). There was a higher proportion of consanguinity in the second-line group (14.8 vs 4.5%; $p < 0.001$).

Molecular genetic results

From 0 to 3 variants were generally observed after filtering, as described.^{7,8} After cosegregation analysis and *in silico* predictions, a causative variant was found in 475 patients

(30.7%) in 35 different genes (Table 2). *SPAST* and *SPG7* were the most frequently mutated genes, representing 142 (9.2% of the whole series) and 75 (4.8%) cases of the series, respectively. The next five most frequently mutated genes (*KIF1A*, *ATL1*, *SPG11*, *KIF5A* and *REEP1*) were involved in more than 17 cases or 1% each (147; 9.5% in total). The remaining 28 genes were more rarely incriminated, affecting 1 (0.06%) to 15 (0.97%) index patients each. No causative variant was detected in 30 of the 65 initially screened genes. Among the 373 patients with the extended screening of seven additional genes, comprising *ABCD1*, *ALDH18A1*, *ATP13A2*, *CAPN1*, *CPT1C*, *LYST* and *PSEN1*, only *ALDH18A1* was found to be carrying a causative variant, and in only one patient (1/373).

Using the panel as a first- or second-line test was not associated with differences in efficacy (28.6% vs 32.9% of patients with causative variants, $p = 0.07$). However, the relative frequencies of gene involvement varied between groups since the patients of the second-line group had firstly been tested for mutations in the major causative genes consistent with the phenotypic presentation (Table 2). Indeed, causative variants in *SPAST* were two times more frequent if the panel was used as first-line test (12.1% vs 5.9%, $p < 0.001$). The third most frequently mutated gene was notably different; in the second-line group it was *KIF1A* (3.4%) whereas in the second-line group it was *ATL1* (2.6%). *ATL1* was the fourth most frequently mutated gene, representing 2.2% of the series. Surprisingly, the frequency of *SPG11* mutations (0.7% to 3.2%) was lower than that previously reported.¹⁰

Forty-six VUS were identified in 37 patients (2.4%) (see Supplementary Tables 2 and 3). They mainly involved *SPAST* ($n = 14$, 37.8%), especially in the first-line group. The genes involved were mainly affected by variants at the heterozygous state: 34/46 variants (73.9%) were located in genes responsible for AD forms. Variants at the heterozygous state in genes reported with AR inheritance only, were considered as not causative when the remaining exons were free of additional variants and in absence of CNV *in trans*.

Inheritance of the causative variants

The probability of finding a pathogenic or likely pathogenic variant was higher when a clear inheritance was determined or at least a familial recurrence (i.e., multiple cases regardless of the mode of inheritance): 44.4% in compatible AD/Dominant transmission (151/340) or 42.2% in AR (65/154) versus 26.6% in undetermined (57/214) or 23.9% in isolated cases (201/840, $p < 0.001$). Mutated genes were mainly associated with AD transmission (280; 58.9%) followed by AR (186; 39.2%) most likely as the result of the continental European

and Mediterranean ancestry of the majority of the cases (Table 2). De novo mutations were identified for 1.7% (n = 8) of all mutated cases: they represented 40% of all *NIPAI* cases (2/5), 10.3% of *KIF1A* cases (4/39), 3.8% of *KIF5A* cases (1/26) and 0.7% of *SPAST* cases (1/142). It must have been underestimated because segregation of variants in both parents was rarely possible in sporadic forms.

We found the usual and main described inheritance modes for most genes. The distribution of genes by inheritance mode is shown in Fig. 1. *SPAST* and *SPG7* were the most frequently involved genes in diagnosed AD or AR forms, representing 58.7% (98/167) and 62.0% (39/64) in the first-line group, respectively. Among the genes with various possible modes of inheritance, *KIF1A* was not involved in AR forms in our series, but it was one of the most frequently mutated genes in AD forms (39/288; 13.5%). Surprisingly, *ERLIN2* was as frequently involved in AR as in AD forms (n = 3 cases of each form) while mainly AR forms have already been described.¹¹ A single X-linked mutation was found, in *PLP1* (0.06% of the series), in a patient presenting with classical Pelizaeus-Merzbacher disease.

Characteristics of the causative variants

The 475 mutated patients carried 661 causative variants (381 different ones) (see Supplementary Table 2, including the additional variant found in a secondary screening in *ALDH18A1*). Thirty of them were structural variants (22 different ones) found in 29 cases: 16 in *SPAST* (Supplementary fig. 1), four in *REEP1*, three in *SPG11*, two in *SPG7*, two in *KIF1A* and one in *ZFYVE26*, all of which were at the heterozygous state, and one full deletion at homozygous state in *DDHD2*. All but one were deletions ranging from one exon to the full gene, the exception being a known duplication of two exons in *SPG11*. There were 631 non-structural variants (359 different). Half of them were likely premature termination codon (PTC) variants (n = 323/631; 51.2%) and the remaining ones were missense variants (n = 308/631; 48.8%). Among all variants, many (n = 252/381, 66.1%) were found as single occurrences in our series and 233 (61.2%) were novel, after an analysis of the literature and ClinVar reports via the VEP tool from Ensembl.¹² Among the recurrent variants, c.1529C>T (p.Ala510Val) in *SPG7* was the most frequent in this study. This variant represented 50 recurrences or 34 cases in *SPG7* (45.3% of variants in this gene). Another recurrent pathogenic variant was c.715C>T (p.Arg239Cys) in *ATL1* representing six cases (17.6%). In eight of the 10 most frequent clinico-genetic entities identified in this series (Table 2), more than 50% of the variants occurred only once in this series: 17/19 (89.5%) for *KIF5A*, 80/103

(77.7%) for *SPAST*, 12/14 (85.7%) for *REEP1*, 26/31 (83.9%) for *KIF1A*, 18/22 (81.8%) for *SACS*, 10/15 (66.7%) for *ATL1*, 4/7 (57.1%) for *WASHC5* and 19/35 (54.3%) for *SPG11*. Among frequent AR forms (> 10 cases), variants at the homozygous state varied from 61.3% (46/75) for *SPG7* to 23.1% (3/13) for *SACS*. All variants at the homozygous state in *SACS* resulted from consanguineous marriages, while consanguinity represented less than 50% in other forms. For *SPG7*, this was explained by the recurrence of the p.Ala510Val variant found in 14/41 (34.1%) of the non-consanguineous patients with a variant at the homozygous state.

Phenotype-genotype correlations

This largest HSP cohort reported so far allowed us to define the phenotypic spectrum of HSP-related disorders and to search for possible gene-related endophenotypes.

The detailed clinical picture in variant carriers, limited to index cases of the most frequent subtypes, is summarized in Supplementary Fig. 2 and Supplementary Table 4. As expected, lower limb spasticity was the most frequent clinical feature, reported in 95.7% (420/439) and 95.3% (937/987) of mutated and not mutated groups, respectively ($p = 0.55$). There were no statistical differences between mutated and not mutated cases in terms of clinical signs, except for superficial sensory impairment or neuropathy at electroneuromyography (ENMG), which was more frequent among mutated cases (respectively 25.9% (52/201) and 15.6% (79/505), $p = 0.002$) and for upper limb spasticity, which was more frequent among not mutated cases (respectively 25.8% (97/376) and 33.6% (282/839), $p = 0.007$).

In variant carriers, age at onset distinguished several entities as early-onset (median onset < 20 years) with *SACS*, *ATL1* and *KIF1A* and late-onset disorders (median onset > 20 years) with *SPAST* and *SPG7*, even if the genes with the more cases showed a variability in age at onset that extended throughout life (Fig. 2). Interestingly, there was no difference related to inheritance, with median age at onset of 23.3 years vs 29.6 years in AR and AD forms, respectively ($p = 0.49$). *DDHD2* (n = 7) patients seemed to have the most severe phenotype, associating an early onset (median of 1.5 years [iqr 1.25]), a median disability score of 6 (iqr 0) and frequent additional signs (100%, 6/6), such as intellectual involvement (100%, 6/6), epilepsy (40%, 2/5), cerebellar ataxia (50%, 1/2) and dysphagia (100%, 1/1). This was also the case for the majority of other AR forms, whereas AD forms were less severe, as observed in most human diseases. Indeed, complicated forms were more frequent

in AR forms (75.7% [140/185] vs 42.0% [121/288], $p < 0.001$). Their median functional disability seemed also higher but not significantly different with a score of 4 [iqr 2] versus 3 [iqr 2] ($p = 0.09$) after similar median disease durations (14.6 years [iqr 23.2] in AD and 16.1 years [iqr 19.4] in AR forms, $p = 0.049$). Thus, the most frequent clinical signs associated with spasticity were also more frequent in AR forms ($p < 0.001$), such as cognitive impairment (36.7%, 61/166 vs 14.7%, 38/259 diagnosed cases with available data). It was also the case for upper limb spasticity, dysphagia, dysarthria, cerebellar ataxia, ocular movement abnormalities, diminished visual acuity, extrapyramidal involvement and abnormal brain MRI. In contrast, epilepsy and superficial neuropathy were not more frequent in AR than in AD forms (1.9% (3/160) vs 3.3% (9/275), $p = 0.55$) (24.1% (20/83) vs 26.5% (31/117), $p = 0.7$).

Except for *REEP2* (one single patient), all the mutated genes identified in our study could be associated with complicated forms, demonstrating that the historical distinction between pure and complicated HSP has a little relevance in diagnosis. However, pure forms are more frequent in *ERLIN2* (83.3%, 5/6), *SPAST* (69.7%, 99/142) and *ATL1* (67.6%, 23/34).

There was no specific sign in any of the clinico-genetic entities that could guide molecular diagnosis. For example, skeletal anomalies were very frequent in *KIF1A* mutation carriers (58.8%, 20/34 mutated cases), but the main cause of skeletal problems was *SPAST* variants, representing 7.8% (33/422) of all the patients with this sign (vs 4.7%, 20/422 for *KIF1A*) because of their higher frequency in our HSP cohort (9.2% for *SPAST* versus 2.5% for *KIF1A*) (Supplementary Tables 5 and 6). Lower limb spasticity, while present in almost all patients, was less frequent in *SACS* mutation carriers (58.3%, 7/12, $p < 0.001$), but this may be explained by an insufficient duration of the disease, which in most patients started with cerebellar dysfunction. Interestingly, 30.5% (110/361) of all patients with cerebellar ataxia were mutated in 25 different HSP genes, the most frequent of these being *SPG7* (9.7% of all cases with cerebellar ataxia, 35/361). Among the genes with more than 10 mutated patients identified, *SACS* had the highest rate of ataxia (100%, 9/9) followed by other AR genes: *SPG7* (57.4%, 35/61), *CYP7B1* (55.6%, 5/9), *SPG11* (53.3%, 8/15) and for AD genes: *KIF5A* (38.9%, 7/18). Peripheral sensory neuropathy (defined by superficial sensory loss and/or by ENMG) was particularly frequent in patients with *KIF1A* or *KIF5A* mutations (55.6%, 10/18 and 78.6%, 11/14, respectively), representing the main cause of neuropathy with *SPG7* (22.2%, 8/36) in the whole series (21.8%, 29/133). *SPAST* and *KIF1A* accounted

respectively for 6.1% and 7.6% (4/66 and 5/66) of facial dysmorphism in the series, although it was more frequent in *KIF1A* cases (18.5%, 5/27). *SACS* was the first cause of diminished visual acuity (8.7%, 2/23). Intellectual involvement rather incriminated *KIF1A* or *SPG11* representing respectively 17/308 and 16/308 (5.5% and 5.2% of cases with the sign in the series), followed by *SPAST* and *SPG7* (3.9% each, 12/308). There were, however, some constellations of signs that were more often associated with a given gene. For example, the association of epilepsy and neuropathy pointed more strongly towards *KIF5A* than each sign alone, but this association was present in only four patients, with two not mutated for the genes screened, and one each mutated in *KIF5A* and *PLP1*. The association of neuropathy and cognitive deficit guided more towards *KIF1A*, which represented 18.5% (5/27 cases) of this association in the series, or towards *KIF5A* (7.4%, 2/27).

Abnormal brain MRI was frequent in *SPG11* (73.7%, 14/19: first, thin corpus callosum but also cortical, brainstem and cerebellar atrophy) and *SACS* (70.0%, 7/10: cerebellar and corpus callosum atrophy). However, the most frequent causes of abnormal brain MRI were causative variants in *SPG7* (8.7%, 33/379, compared to 3.7% [14/379] for *SPG11* and 1.8% [7/379]) for *SACS*, with cerebellar atrophy), as a result of their high frequency in our cohort. In contrast, brain MRI abnormalities were rare in *SPAST*, *ERLIN2* and *AP5Z1* variant carriers.

Recurrent mutations of the same gene identified in multiple families showed a wide inter-familial phenotypic variability. For example, intellectual involvement was found in only 1/7 cases (14.3%) and extrapyramidal signs in 3/6 (50.0%) for c.839G>A (p.Arg280His), the most recurrent variant of *KIF5A*. Another example of clinical heterogeneity was the intellectual involvement in only two (22.2%) of the nine patients with *SPAST* exon deletions leading to frameshifts.

SPAST, *SPG7* and *KIF1A* patients presented with clinico-genetic characteristics similar to previous reports,^{10,13} including some of our patients already reported in large cohorts.^{14,15} There was no significant link between the position of the pathogenic variant in the protein and the phenotype when we focused on the 10 most frequently mutated genes (involving more than 10 patients diagnosed).

Interestingly, this large series allowed us extending the phenotype associated with rare genetic entities. For example, our two *DDHD2* patients homozygous for the recurrent p.Asp660His and the private PTC p.Arg329Ter variant, respectively, had early epilepsy, a

clinical feature never previously reported in this form. Regarding the *KIF5A* gene, we identified 2/26 patients with epilepsy (7.7%) carrying the p.Asn256Ser and p.Pro278Leu heterozygous variant, thus expanding the epilepsy phenotype to missense variants, previously associated only with PTC variants.¹⁶ We also reported the first *KIF1C*-related cases, with intellectual disability in 2 out of 3 mutated patients, extending the known *SPG58* phenotype. One had the c.1019dupG variant affecting splicing at the homozygous state and the second was compound heterozygous for c.1346A>G and c.2920G>A missense mutations. Brain MRIs of six patients carrying variants in *WASHC5* (out of the 10 available) were abnormal, with cerebellar atrophy and white matter hyperintensities classifying them as complex forms. Only two MRIs were reported as abnormal in the latest literature review of *SPG8* cases (23 families), with white matter hyperintensities and spinal cord atrophy.¹⁷

In other clinico-genetic entities, the mutation spectra were clarified, such as in *REEP1*, in which we found a significant frequency of structural variants (22.2%, 4/18) compared to previous reports, which mainly did not search specifically for genomic rearrangements. The detection of rearrangements is, therefore, crucial for *REEP1* molecular diagnosis. However, clinical characteristics did not differ from those described in the literature. We also identified two new AD *ERLIN2* missense mutations in the SPFH domain, while variants in AR forms were always frameshift variants or in the oligomerization domain,¹¹ suggesting, therefore, phenotype-genotype correlations in this form. Finally, in *KIF1A*, frameshift variants had recently been reported as pathogenic at the heterozygous state with de novo cases.¹³ Here, we confirm this finding and interpreted two heterozygous exonic deletions and two heterozygous splice variants highly predicted as deleterious, as pathogenic variants.

Exome sequencing

In a small series of 42 negative index cases from this study, subsequent WES analysis allowed the identification of variants in 11 of them (26%, Supplementary table 7) in genes from the Online Mendelian Inheritance in Man catalogue (<https://www.omim.org/>). Five of these variants were identified in recently reported HSP genes (*CAPN1* n=4, *RNF170* n=1), in an intronic region of a known HSP gene (*SPAST*, n=1) or in genes associated with clinical syndromes in which spasticity is part of a large clinical spectrum (*ERCC5*, *TCF20*, *HACE1*, *VRK1*, *PLA2G6*). The phenotypic presentation fitted at least partially the known clinical presentation. Of note, one VUS was identified in *SLC19A3*, encoding a thiamine

transmembrane transporter, but as it alters only one isoform of the gene and since biochemical dosage of CSF metabolites of thiamine are not possible in the index case, we were not able to conclude on the causative nature of the variant.

Discussion

Our series of HSP patients was typical from real-life molecular diagnosis and also included a smaller, non-overlapping cohort from a research context. With 30.7% of patients with pathogenic or likely pathogenic variant(s), we confirm the diagnostic efficiency of HSP-targeting sequencing panels as a first-line genetic test. This is within the range (29-32%) reported in the literature in similar diagnosis cohorts,^{18,19} but lower than in research-focused series.^{20,21} In addition, early onset age, consanguinity, dominant transmission or familial recurrence are factors that increase the power of NGS in HSP diagnosis, as also shown by others.⁶ We found fewer VUS (2.4%) compared to the 36% observed in a similar Italian HSP cohort analysis using a targeted gene panel.¹⁸ The number of VUS can increase with the number of tested genes, which was the case in the Italian panel (n = 118). Moreover, segregation of variants in relatives was effective in our series to reduce the number of VUS and in addition, we did not consider as VUS variants at the heterozygous state in AR genes when the gene was successfully covered and when no CNV was detected, except in few cases such as *KIF1A*, *ERLIN2* and *SPG7*, based on previous literature findings. We do not exclude that more variants at the heterozygous state in AR genes may be causative in the future, therefore becoming a clear diagnosis issue. The existence of VUS variants calls for the development of functional tests for their biological validation in vitro or in vivo since biomarkers are most of the time lacking in HSP.^{2,6}

Gene frequency

SPAST is the most frequently involved gene in HSP and accounts for the most frequent AD form, SPG4. Interestingly, *KIF1A* variants have also been suggested as a frequent AD form in recent years and we confirm this finding in a large cohort for the first time. Among recessive forms, *SPG7* is the most frequent clinical entity. *SPG11* was found less frequently than in previous reports, which focused on patients fitting the recognizable clinical features, including mental impairment, neuropathy and thin corpus callosum.¹⁰

The vast majority of the diagnosed cases was obtained in a small number of genes (76.6% involving 10 genes). The rarer genes tested here affected 29/1550 cases (1.9% of the series or 6.1% of the diagnosed patients), with fewer than four cases by gene. However, one interest of NGS panels is to enable the rarest causative genes to be diagnosed as well thanks to the large number of genes screened in a single test. It is particularly suitable for rare genetic diseases like HSP with a large number of genes involved.^{5,6} However, the increasing number of HSP forms overlapping other neurological phenotypes and of HSP-associated genes makes it difficult to keep the panels up to date. Of note, no causative variants were found in 30 of the screened genes in our large cohort, questioning the interest of maintaining them in future versions of the HSP panel in France. Some of them (*SAMHD*, *CCT5* and *GADI*, for example) are reported in patients with syndrome in which spastic paraplegia is only part of a more complex phenotype. The number of undiagnosed cases due to non-updated versions of panels would therefore be very low if these very rarely involved genes were omitted. Indeed, the additional screening of seven new rare HSP genes in 373 of our patients identified only one case mutated in *ALDH18A1*. These patients could also be diagnosed by another clinical entry, such as intellectual deficiency.

Nature of the variants

The great number of novel variants in our screening (n = 233 [61.2%]) fits with the wide molecular variability of HSP but was most likely overestimated due to the delay in updating databases. Nevertheless, many variants were found with a single occurrence in our large series.

Rearrangements are not rare in HSP genes, particularly in *SPAST*: 11.3% (16/142 patients) in our series and up to 20% in previous studies.²³ Beyond punctual or small indel variations, our panel also enabled us to detect structural rearrangements in genes via a specific algorithm based on read depth analysis. This method can detect single exon deletions as well as small duplications. This NGS-panel capability was not reported in the large Italian series¹⁸ and only once in our previous report.⁷ The detection of structural variants is critical in HSP as they occurred in 29/475 (6.1%) of the mutated cases (16 [6.9%] in the first-line group and 13 [5.3%] in the second-line group). In particular, we underline the need to search for *REEP1* rearrangements (22.2%, 4/18).²⁴

Factors influencing the yield

Classification of HSP partly relies on inheritance, but inheritance does not always help to guide gene identification. Indeed, the mode of inheritance is difficult to define in many conditions: i) low penetrance, ii) absence of familial history, iii) small size of the pedigree, iv) insufficient genealogical assessment by the clinician. Moreover, different modes of inheritance can be associated with the same gene. This was seen in the 50% AR and 50% AD transmission for *ERLIN2* variants in our cohort. De novo variants also hide the mode of inheritance for the first generation. This was particularly notable for two (40%) of the five variants in *NIPAI1*.

Lower limb spasticity is a hallmark of HSP. However, because of overlapping phenotypes and clinical variability, HSP-related genes are involved more broadly in ataxia-spasticity spectrum disorders.⁵ This explains why lower limb spasticity was not always reported in our cases (95.3% in patients with available data) and illustrates the clinical continuum of HSP-related disorders. However, all our patients had pyramidal signs. Many clinical features can be shared by different genes involved in these diseases and, as exemplified in our study, each novel case is the potential source of enlargement of the phenotypic spectrum related to a given gene. Thus, it is impossible to predict the mutated gene from the neurological examination even if frequency, mode of inheritance and age at onset are the most relevant criteria.

Panel of genes versus whole exome sequencing

The use of a panel of genes fitted well the routine diagnosis in clinical practice since preventing the incidental findings, facilitating variant and CNV identification in a series of genes focused for the HSP diagnosis prescription. Most of the mutations were found in a restricted number of genes, which then suggest the use of genetic panels in first-line tests to accelerate genetic diagnosis that may be restricted to the 35 genes found mutated in our French cohort. On the other hand, our resequencing HSP panel prevented the identification of novel causative genes, which is however rarely possible in routine diagnosis because of family size and uneasy access to functional studies. We then suggest to use a two-steps strategy using genetic panels followed by WES. Indeed, in a small series of 42 negative cases from this study, subsequent WES allowed the identification of variants in 11 of them (26%), five of them were published elsewhere.^{25,26} This two-steps strategy would then increase the diagnosis yield up to 48.8% of HSP cases.

Conclusion

We have reported the largest real-life experience of routine molecular diagnosis in HSP. The use of a targeted multigene panel on 1550 patients led to ~31% of solved diagnosis. Our panel enabled screening of a large number of HSP-related genes in a single test, a good solution to look for rarer involved genes in large cohorts, even if as shown here most mutations are concentrated in a small number of causative genes (77% in 10 genes). Even if the cost of exome and targeted genes panels is now similar in terms of consumables, our NGS panel could also detect structural rearrangements via coverage analysis, which is less efficient in current exome data sets. These structural variants represent a significant part of the pathogenic HSP variants (6.1% of the patients). This is the reason why we favour a two-step approach using the multigene panel and then WES or WGS in negative families, which together increase the diagnosis rate up to ~50%. The current diagnostic panel of genes should probably be optimized according to the results observed here by removing the 30 genes with no causative variants found in France and adding the novel genes found mutated in exome sequencing, particularly the recurrent *CAPN1*. However, in patients with a syndromic form of a neurological disorder in which HSP is only a symptom of a large number of other clinical signs that do not fit one SPG gene (i.e. *SPG11*), WES or WGS may be used directly and when possible using trios to facilitate the identification of *de novo* cases by expert centres applying adequate phenotypic/genetic filters.

Continuing to discover new variants in HSP-related genes and to describe more patients is important so as to encompass the wide phenotype associated and to give a diagnosis to patients and their families, given that ~50% of the cases still have no known aetiology.

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Figure legends

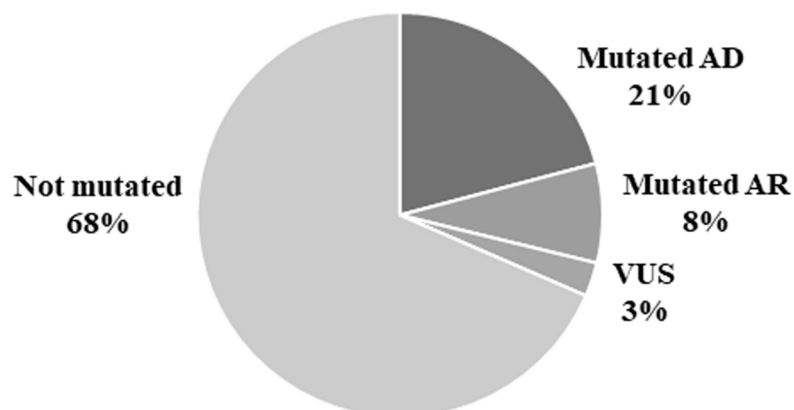
Figure 1 Distribution of the mutated genes according to transmission mode in 809 index patients with first-line analysis

AD: autosomal dominant; AR: autosomal recessive

Figure 2 Boxplots of age at onset according to the gene carrying variants and inheritance mode

Autosomal dominant in orange and autosomal recessive in blue; 25 and 75th percentile range represented by the boxes; horizontal black line for the median onset (50th percentile) and vertical line showing the 10th and 90th percentiles; dots for each value.

Gene panel results in the first-line group (n = 809)



Causative genes in the first-line group (n = 231)

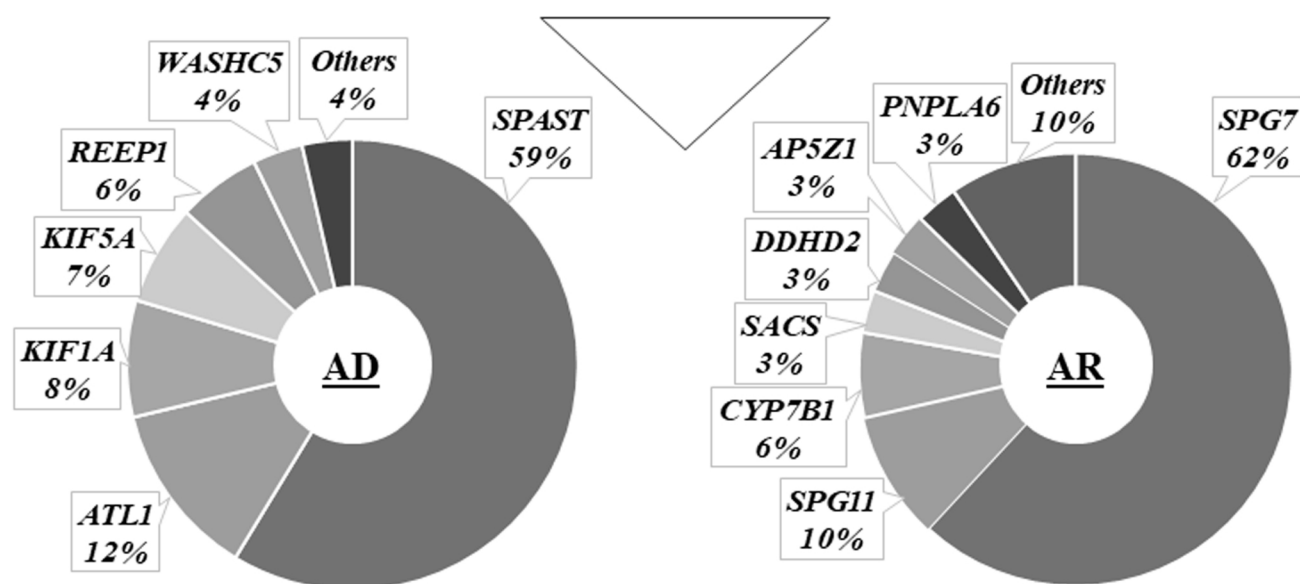


FIGURE 1

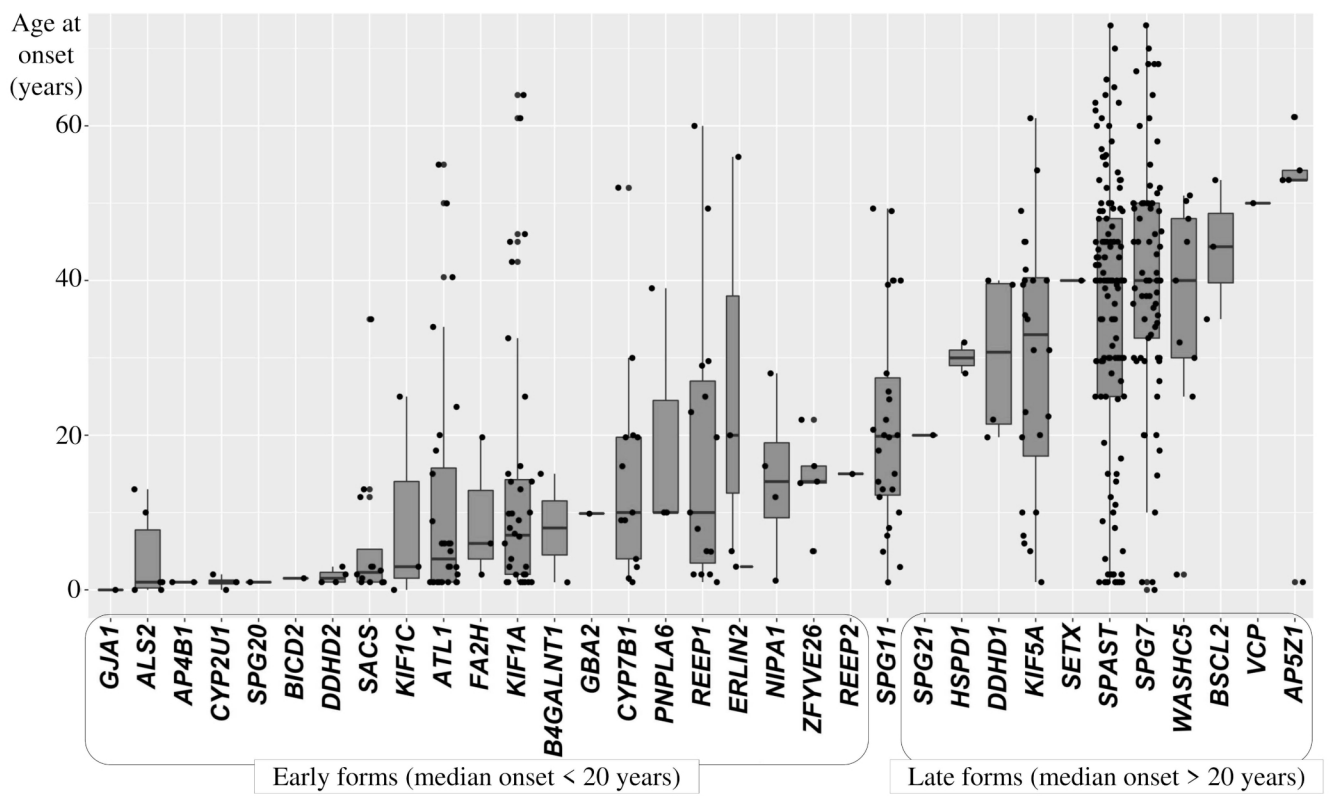


FIGURE 2

Table 1 General characteristics of the cohort

	All patients (n = 1550)	First-line (n = 809)	Second-line (n = 741)	p^a
Suspected mode of inheritance				<0.001
Isolated case	840 (54.2%)	517 (63.9%)	323 (43.6%)	<0.001
Autosomal dominant/Dominant	340 (21.9%)	149 (18.4%)	191 (25.8%)	<0.001
Autosomal recessive	154 (9.9%)	36 (4.5%)	118 (15.9%)	<0.001
X-linked	2 (0.1%)	2 (0.25%)	0 (0%)	0.5
Unknown	214 (13.8%)	105 (13%)	109 (14.7%)	0.36
Gender				0.27
Male	854 (55.1%)	452 (55.9%)	402 (54.3%)	0.56
Female	688 (44.4%)	355 (43.9%)	333 (44.9%)	0.71
Unknown	8 (0.5%)	2 (0.2%)	6 (0.8%)	0.16
Phenotype				0.45
Pure	680 (43.9%)	362 (44.7%)	318 (42.9%)	0.5
Complicated	869 (56.1%)	447 (55.3%)	422 (57.0%)	0.53
Unknown	1 (0.1%)	0 (0%)	1 (0.1%)	0.48
Age at onset (median year / iqr)	25 (40.8)	32 (45)	20 (36.1)	<0.001
Age at examination (mean year / iqr)	46.4 (31)	48.5 (32)	43.5 (29.6)	0.03
Disease duration (mean year / iqr)	12.4 (20.8)	10.3 (19.8)	14.7 (21.6)	0.004
Severity ^b (median stage 0-7 / iqr)	3 (2)	3 (2)	3 (2)	0.35

iqr= interquartile range.

^aDifference between the first- and second-line groups with p for significance value.

^bSPATAX functional score ranging from 0 (no disability) to 7 (severe disability/confined to bed) according to <https://spatax.files.wordpress.com/2013/09/fichecliniquespatax-eurospa-2011.pdf>.

Table 2 Genetic results for the 65 genes of the initial screening according to inheritance model

Patients with:		All patients (n = 1550)	First-line (n = 809)	Second-line (n = 741)	p ^a
No causative variant		1038 (66.97%)	554 (68.48%)	484 (65.32%)	0.2
VUS		37 (2.39%)	24 (2.97%)	13 (1.75%)	0.16
Causative variant		475 (30.65%)	231 (28.55%)	244 (32.93%)	0.07
<i>SPAST</i>	SPG4	142 (9.16%)	98 (12.11%)	44 (5.94%)	<0.001
<i>SPG7</i>	SPG7	75 (4.84%)	39 (4.82%)	36 (4.86%)	0.52
<i>KIF1A</i>	SPG30	39 (2.52%)	14 (1.73%)	25 (3.37%)	0.1
<i>ATL1</i>	SPG3A	34 (2.19%)	21 (2.6%)	13 (1.75%)	0.11
<i>SPG11</i>	SPG11	30 (1.94%)	6 (0.74%)	24 (3.24%)	0.001
<i>KIF5A</i>	SPG10	26 (1.68%)	12 (1.48%)	14 (1.89%)	0.79
<i>REEP1</i>	SPG31	18 (1.16%)	10 (1.24%)	8 (1.08%)	0.55
<i>CYP7B1</i>	SPG5A	15 (0.97%)	4 (0.49%)	11 (1.48%)	0.08
<i>SACS</i>		13 (0.84%)	2 (0.25%)	11 (1.48%)	0.02
<i>WASHC5</i>	SPG8	12 (0.77%)	6 (0.74%)	6 (0.81%)	0.92
<i>DDHD2</i>	SPG54	7 (0.45%)	2 (0.25%)	5 (0.67%)	0.45
<i>ALS2</i>		6 (0.39%)	1 (0.12%)	5 (0.67%)	0.22
<i>ERLIN2</i>	SPG18	6 (0.39%)	1 (0.12%)	5 (0.67%)	0.22
<i>AP5Z1</i>	SPG48	5 (0.32%)	2 (0.25%)	3 (0.4%)	1
<i>ZFYVE26</i>	SPG15	5 (0.32%)	0 (0%)	5 (0.67%)	0.06
<i>NIPA1</i>	SPG6	5 (0.32%)	1 (0.12%)	4 (0.54%)	0.37
<i>DDHD1</i>	SPG28	4 (0.26%)	0 (0%)	4 (0.54%)	0.12
<i>CYP2U1</i>	SPG56	4 (0.26%)	1 (0.12%)	3 (0.4%)	0.62
<i>BSC12</i>	SPG17	3 (0.19%)	2 (0.25%)	1 (0.13%)	0.61
<i>KIF1C</i>	SPG58	3 (0.19%)	0 (0%)	3 (0.4%)	0.25
<i>FA2H</i>	SPG35	3 (0.19%)	0 (0%)	3 (0.4%)	0.25
<i>PNPLA6</i>	SPG39	3 (0.19%)	2 (0.25%)	1 (0.13%)	0.61
<i>GBA2</i>	SPG46	2 (0.13%)	0 (0%)	2 (0.27%)	0.5
<i>B4GALNT1</i>	SPG26	2 (0.13%)	1 (0.12%)	1 (0.13%)	1
<i>AP4B1</i>	SPG47	2 (0.13%)	1 (0.12%)	1 (0.13%)	1
<i>HSPD1</i>	SPG13	2 (0.13%)	0 (0%)	2 (0.27%)	0.5
<i>BICD2</i>		1 (0.06%)	0 (0%)	1 (0.13%)	1
<i>MARS</i>	SPG70	1 (0.06%)	0 (0%)	1 (0.13%)	1
<i>PLP1</i>	SPG2	1 (0.06%)	1 (0.12%)	0 (0%)	0.49
<i>REEP2</i>	SPG72	1 (0.06%)	1 (0.12%)	0 (0%)	0.49
<i>SPG20</i>	SPG20	1 (0.06%)	1 (0.12%)	0 (0%)	0.49
<i>SPG21</i>	SPG21	1 (0.06%)	1 (0.12%)	0 (0%)	0.49
<i>VCP</i>		1 (0.06%)	1 (0.12%)	0 (0%)	0.49
<i>GJA1</i>		1 (0.06%)	0 (0%)	1 (0.13%)	1
<i>SETX</i>		1 (0.06%)	0 (0%)	1 (0.13%)	1
Variant transmission					
De novo (AD)		8 (1.7%)	1 (0.4%)	7 (2.9%)	0.06
AD/Dominant		280 (58.9%)	166 (71.9%)	114 (46.7%)	0.01
AR		186 (39.2%)	63 (27.3%)	123 (50.4%)	<0.001
XL		1 (0.2%)	1 (0.4%)	0 (0%)	1

^aDifference between the first- and second-line groups with p for significance value.

AD: autosomal dominant; AR: autosomal recessive; XL: X-linked