Next-generation sequencing study reveals the broader variant spectrum of hereditary spastic paraplegia and related phenotypes

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Abstract
Hereditary spastic paraplegias (HSPs) are clinically and genetically heterogeneous neurodegenerative disorders. Numerous genes linked to HSPs, overlapping phenotypes between HSP subtypes and other neurodegenerative disorders and the HSPs’ dual mode of inheritance (both dominant and recessive) make the genetic diagnosis of HSPs complex and difficult. Out of the original HSP cohort comprising 306 index cases (familial and isolated) who had been tested according to traditional workflow/guidelines by Multiplex Ligation-dependent Probe Amplification (MLPA) and Sanger sequencing, 30 unrelated patients (all familial cases) with unsolved genetic diagnoses were tested using next-generation sequencing (NGS). One hundred thirty-two genes associated with spastic paraplegias, hereditary ataxias and related movement disorders were analysed using the Illumina TruSight™ One Sequencing Panel. The targeted NGS data showed pathogenic variants, likely pathogenic variants and those of uncertain significance (VUS) in the following genes: SPAST (spastin, SPG4), ATL1 (atlastin 1, SPG3), WASHC5 (SPG8), KIF5A (SPG10), KIF1A (SPG30), SPG11 (spatacsin), CYP27A1, SETX and ITPR1. Out of the nine genes mentioned above, three have not been directly associated with the HSP phenotype to date. Considering the phenotypic overlap and joint cellular pathways of the HSP, spinocerebellar ataxia (SCA) and amyotrophic lateral sclerosis (ALS) genes, our findings provide further evidence that common genetic testing may improve the diagnostics of movement disorders with a spectrum of ataxia-spasticity signs.

Keywords Ataxia-spasticity · Hereditary spastic paraplegia · Movement disorders · Next-generation sequencing.

Introduction
Hereditary spastic paraplegias (HSPs) comprise a group of genetic disorders resulting from neurodegeneration of the corticospinal tracts. The HSPs’ main clinical feature is progressive spasticity and weakness of the lower limbs. HSP is classified as a pure form when symptoms are limited to: progressive spasticity and weakness of the lower limbs, bladder dysfunction and mild somatosensory deficits. In case of any additional neurological symptoms, a complicated HSP form is recognised. To date, over 70 different SPG loci have been identified, and over 60 corresponding genes have been investigated [1–3]. All modes of HSP inheritance have already been described: autosomal dominant (ADHSP), autosomal recessive (ARHSP), X-linked (XLHSP) and less frequently, mitochondrial. Among 20 different ADHSP subtypes, SPG4 is the most common one, accounting for approximately 40% of the cases. The frequency of other ADHSP subtypes ranges from 1% to 10%. The main ARHSPs identified to date are SPG5, SPG7, SPG11 and SPG15 [4].
According to population studies, the proportion of families without genetic diagnosis ranged from 45% to 67% in the ADHSP and from 71% to 82% in the ARHSP groups [5]. Recently reported dual-transmission of some HSP subtypes makes their molecular characterisation even more complicated. Due to the HSP heterogeneity, next-generation sequencing (NGS) became a highly useful screening tool in HSP investigations and differential diagnosis. Broad NGS studies have revealed a clinical and genetic overlap between different HSP subtypes, as well as between other neurodegenerative disorders, such as hereditary spinocerebellar ataxias (SCAs), amyotrophic lateral sclerosis (ALS) and neuropathies [6].

In the present study, we analysed familial HSP patients through spastic-ataxia spectrum disease genes according to the approach suggested by Synofzik et al. [6].

Materials and methods

The study was approved by the Bioethics Committee of the Institute of Psychiatry and Neurology in Warsaw. All of the participants provided informed consent.

In the presented study, we aimed to test a group of 30 unrelated hereditary spastic paraplegia patients using the targeted Illumina TruSight™ One Sequencing Panel (Illumina). The original HSP cohort comprised 306 probands in which Multiplex Ligation-dependent Probe Amplification (MLPA) and Sanger Sequencing had been performed to diagnose five HSP subtypes (SPG3, SPG4, SPG6, SPG11 and SPG31) in 62 families [7–10]. Out of the remaining 244 probands, 30 familial HSP index cases were selected for NGS testing. The major inclusion criteria comprise: (i) spastic paraplegia as a main clinical feature, (ii) positive family history and (iii) availability of DNA sample for more than one affected family member and/or potential carriers. The families’ history suggested AD inheritance in 18 and AR in 12 families. In three probands, SPG11 deletions and duplication had been identified in one allele, and NGS sequencing focused on searching for the second causative variant to confirm the AR SPG11. One identified carrier of the SPAST pathogenic variant was used as a positive control in the NGS screening (Fig. 1).

All studied patients were evaluated according to the Fink criteria for HSP [11]. The HSP pure form was observed in 16 probands, and the complicated form was observed in 14 probands.

The Illumina TruSight™ One Sequencing Panel covering the coding regions of the 4813 genes associated with the known clinical phenotypes was used (https://www.illumina.com/products/by-type/clinical-research-products/trusight-one.html). The library preparation, labelling and enrichment were performed according to the protocol using 50 ng of DNA input. The coding regions of 132 genes linked to spastic paraplegias, hereditary ataxias and related movement disorders were analysed. The data were analysed using Illumina VariantStudio 2.2 and visualised in Integrated Genomics Viewer (IGV) (Broad Institute). To investigate the evolutionary conservation score (PhyloP) and functional prediction of identified mutations, we used SIFT (http://sift.jcvi.org/), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/) and Alamut software (http://www.interactive-biosoftware.com/), as well as the dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) and ClinVar databases (https://www.ncbi.nlm.nih.gov/clinvar/).

NGS data were filtered according to the following criteria: (i) read depth higher than 20 reads and variant frequency higher than 25%; (ii) variants reported less frequently than 0.005 in the Exome Aggregation Consortium database (http://exac.broadinstitute.org/); and (iii) exclusion of all the synonymous and deep intronic variants.

The bioinformatically analysed 132 ataxia-spasticity panel genes involved the following: (1) 37 genes directly linked with HSP: 12-ADHSP, 22-ARHSP and 3-XLHSP; (2) 25 genes linked with hereditary ataxias: 12 AD spinocerebellar ataxia (SCA), 11 ARSCA (SCAR) and four spastic-ataxia (SPAX) genes; (3) three leucodystrophy genes; (4) 14 amyotrophic lateral sclerosis (ALS) genes; (5) 16 genes linked with different neuropathies, including five hereditary motor neuropathies (HMN) and six Charcot Marie-Tooth neuropathies; and (6) other complex movement or multisystem disorders with prominent gait disturbances, comprising 42 genes (Supplementary Table 1). Because certain genes are linked with more than one phenotype, the number of genes and conditions are not equal. The classification and interpretation of the identified variants were performed according to recommendations of the American College of Medical Genetics and Genomic and the Association for Molecular Pathology (ACMG&AMP) (Table 1) [12]. Variants selected through filtering were confirmed by Sanger sequencing in the probands and their family members.

Results

The NGS TruSight™ One output data reached approximately 97% of the aligned reads. A mean number of 16,752,119 reads with 259 base pair length fragments per sample was obtained. An average of 91.2% of targeted reads passed the Q score, whereas 88% were covered at least 30 times.
In this study, we identified 18 pathogenic and likely pathogenic variants in 16 spastic paraplegia probands, as well as six variants of uncertain significance (Table 2; Table 3). The most frequent HSP genetic types, SPG4 and SPG3, were identified in five probands: SPAST (SPG4) pathogenic variants in three probands and ATL1 (SPG3) in two probands. In four of the mentioned probands, a previous study involved only the MLPA screening, and one of the SPG4 patients was known to carry a pathogenic variant. In 11 out of 22 individuals, in whom SPAST, ATL1 and REEP1 gene single nucleotide variants (SNV) were previously excluded by Sanger sequencing, we identified three HSP subtypes with AD transmission: WASHC5 (SPG8), KIF5A (SPG10) and KIF1A (SPG30) and SPG11 as the only ARHSPs. Moreover, in one case, a homozygous variant in the CYP27A1 gene, known as pathogenic in cerebrotendinous xanthomatosis (CTX), was identified. Among six variants of uncertain significance we detected: WASHC5, KIF5A, SETX and ITPR1 variants in families with AD mode of inheritance. We were not able to detect any variant corresponding to phenotype in 27% of the examined cohort (four cases with AD and four with AR mode of inheritance).

### Autosomal dominant HSPs

#### ATL1 (SPG3)

One known pathogenic ATL1 variant: c.715C>T (p.Arg239Cys) and one novel: c.1064A>C (p.Asn355Thr) were identified in two HSP probands. The variants presented pure HSP with the age of onset at the first and second years of life.

#### SPAST (SPG4)

In the SPAST gene, the variants were identified in three probands: a missense (c.1378C>T-p.Arg460Cys), nonsense (c.1597G>T-p.Glu533*) and splice site (c.1617-2A>G) mutation. SPAST c.1378C>T is a known pathogenic variant, a moderately conserved nucleotide and highly conserved amino acid position. The two other SPAST gene variants (c.1597G>T and c.1617-2A>G) have not been previously described, neither in the patient cohorts nor in population studies. The ages at onset in the three SPG4 patients were 35, 42 and 28 years,
| Patient ID | Gene | cDNA change | ACMG criteria | ACMG classification |
|------------|------|-------------|---------------|---------------------|
| SPG0902    | ATL1 | NM_015915.4:c.715C>T | PM1 + PM2 + PP1 + PP3 + PP4 + PP5 | Likely pathogenic |
|            |      | NP_050999.2:p.(Arg239Cys) |               |                     |
| SPG0901    | ATL1 | NM_015915.4:c.1064A>C | PM1 + PM2 + PP3 + PP4 | Likely pathogenic |
|            |      | NP_050999.2:p.(Asn355Thr) |               |                     |
| SPG1301    | SPAST| NM_014946.3:c.1378C>T | PM1 + PM2 + PP4 + PP3 + PP5 | Likely pathogenic |
|            |      | NP_055761.2:p.(Arg460Cys) |               |                     |
| SPG0102    | SPAST| NM_014946.3:c.1597G>T | PVS1 + PM2 + PM4 + PM5 + PP4 | Pathogenic |
|            |      | NP_055761.2:p.(Glu533*) |               |                     |
| SPG1401    | SPAST| NM_014946.3:c.1617-2A>G | PVS1 + PM2 + PP4 | Pathogenic |
| SPG0403    | WASHC5| NM_014846.3:c.647C>T | PP1 + PP3 + PP4 | Uncertain significance |
|            |      | NP_055661.3:p.(Pro216Leu) |               |                     |
| SPG0302    | WASHC5| NM_014846.3:c.1859T>C | PM2 + PP1 + PP3 + PP4 + PP5 | Likely pathogenic |
|            |      | NP_055661.3:p.(Val620 Ala) |               |                     |
| SPG0201    | KIF5A | NM_004984.2:c.484C>T | PM1 + PP3 + PP4 + PP5 | Likely pathogenic |
|            |      | NP_004975.2:p.(Arg162Trp) |               |                     |
| SPG1402    | KIF5A | NM_004984.2:c.1402C>T | PP3 + PP4 | Uncertain significance |
|            |      | NP_004975.2:p.(Arg468Trp) |               |                     |
| SPG1101    | KIF1A | NM_001244008.1:c.962G>A | PM1 + PM2 + PM4 + PP3 | Likely pathogenic |
| SPG0601    | SPG11| NM_025137.3:c.408_428del | PM2 + PM4 + PP4 + PP5 | Likely pathogenic |
|            |      | NP_079413.3:p.(Glu136_Ile143del) |               |                     |
| SPG1002    | SPG11| NM_025137.3:c.3075insA | PVS1 + PM2 + PP5 | Pathogenic |
|            |      | NP_079413.3:p.(Glu1026Argfs*4) |               |                     |
| SPG1003    | SPG11| NM_025137.3:c.733_734del | PVS1 + PM2 + PM3 + PP5 | Pathogenic |
|            |      | NP_079413.3:p.(Met245Valfs*2) |               |                     |
| SPG0702    | SPG11| NM_025137.3:c.1457-2A>G | PVS1 + PM2 + PM4 + PM3 + PP5 | Pathogenic |
| SPG0502    | SPG11| NM_025137.3:c.5623C>T | PVS1 + PM2 + PM3 + PP5 | Pathogenic |
|            |      | NP_079413.3:p.(Gln1875*) |               |                     |
| SPG0301    | SPG11| NM_025137.3:c.1275insA | PVS1 + PM4 + PP4 + PP5 | Likely pathogenic |
|            |      | NP_079413.3:p.(Glu934Asp) |               |                     |
| SPG0103    | SPG11| NM_025137.3:c.2849delT | PVS1 + PM2 + PM4 | Pathogenic |
|            |      | NP_079413.3:p.(Leu950Trpfs*13) |               |                     |
| SPG0701    | CYP27A1| NM_000784.3:c.379C>T | PM2 + PP4 + PP3 + PP5 | Likely pathogenic |
|            |      | NP_000775.1:p.(Arg127Trp) |               |                     |
| SPG0303    | ITPR1| NM_00116827.1:c.2687C>T | PP1 + PP3 | Uncertain significance |
|            |      | NP_001161744.1:p.(Ala896Val) |               |                     |
| SPG0401    | ITPR1| NM_00116827.1:c.2687C>T | PP1 + PP3 | Uncertain significance |
|            |      | NP_001161744.1:p.(Ala896Val) |               |                     |
| SPG1203    | ITPR1| NM_00116827.1:c.3412A>G | PP3 | Uncertain significance |
|            |      | NP_001161744.1:p.(Met1138Val) |               |                     |
| SPG0503    | SETX | NM_015046.5:c.7417C>T | PP1 + PP3 | Uncertain significance |
|            |      | NP_055861.3:p.(Leu2473Val) |               |                     |

PVS very strong evidence of pathogenicity, PS strong evidence of pathogenicity, PM moderate evidence of pathogenicity, PP supporting evidence of pathogenicity
| Patient ID | Gene   | Chr | Genomic position | cDNA change (protein change) | Allele zygosity | PhyloP score | ClinVar | SIFT/ PolyPhen/ MutTaster | ExAC allele frequency† | rs number | Inheritance |
|-----------|--------|-----|------------------|-----------------------------|-----------------|--------------|---------|--------------------------|------------------------|------------|-------------|
| SPG0902   | ATL1   | 14  | g.51080061       | NM_015915.4:c.715C>T        | ht              | 1208         | Pathogenic | del/ps_dam/dc             | 0                      | rs119476046 | AD          |
| SPG0901   | ATL1   | 14  | g.51089911       | NM_015915.4:c.1064A>C       | ht              | 4.81         | Pathogenic | del/ps_dam/dc             | 0                      | na         | AD          |
| SPG1301   | SPAST  | 2   | g.32362002       | NM_014946.3:c.1378C>T       | ht              | 2754         | Pathogenic | del/ps_dam/dc             | 0                      | rs878854990 | AD          |
| SPG0102   | SPAST  | 2   | g.32368465       | NM_014946.3:c.1597G>T       | ht              | 5131         | na/na/dc | del/ps_dam/dc             | 0                      | na         | AD          |
| SPG1401   | SPAST  | 2   | g.32370004       | NM_014946.3:c.1617-2A>G     | ht              | 3963         | na/na/dc | na/na/dc                 | 0                      | na         | AD          |
| SPG0302   | WASHC5 | 8   | g.12609814       | NM_014846.3:c.1859T>C       | ht              | 5107         | na/na/dc | del/ps_dam/dc             | 0                      | na         | AD          |
| SPG0201   | KIF5A  | 12  | g.57958739       | NM_004984.2:c.484C>T        | ht              | 1838         | na/na/dc | del/ps_dam/dc             | 0 (0.0000083)          | rs748551786 | AD          |
| SPG101    | KIF1A  | 2   | g.247131675      | NM_0001244008.1:c.962G>A    | ht              | 5425         | na/na/dc | del/ps_dam/dc             | 0                      | na         | AD          |
| SPG0601   | SPGII  | 15  | g.44952643       | NM_025137.3:c.408_428del    | c_ht            | 1.28‡        | Pathogenic | na/na/dc                 | 0                      | rs312262714 | AR          |
|           |        |     |                  | NP_079413.3:p.(Glu136_Ile143del) |                |              |          |                          |                        |            |             |
|           |        |     |                  | g.44905697                  | na              | Pathogenic | na/na/dc | 0.0000083 (0.0000083)     | rs312262752          |            |             |
| SPG1002   | SPGII  | 15  | g.44949427       | NM_025137.3:c.733_734del    | c_ht            | 0.23‡        | Pathogenic | na/na/dc                 | 0 (0.0000083)          | rs312262720 | AR          |
|           |        |     |                  | NP_079413.3:p.(Met245Valfs*2) |                |              |          |                          |                        |            |             |
|           |        |     |                  | g.44941193                  | na              | Pathogenic | na/na/dc | 0.000045 (0.000107)       | rs312262727          |            |             |
|           |        |     |                  | NP_079413.3:p.(Leu491Ilefs*66) |                |              |          |                          |                        |            |             |
|           |        |     |                  | g.44859744                  | 0.952           | us          | tol/bn/dc | 0.00127 (0.0008)          | rs144165094          |            |             |
| SPG1003   | SPGII  | 15  | g.44941193       | NM_025137.3:c.1471_1472del  | c_ht            | 2.12‡        | Pathogenic | na/na/dc                 | 0 (0.0000083)          | rs312262727 | AR          |
|           |        |     |                  | NP_079413.3:p.(Leu491Phefs*66) |                |              |          |                          |                        |            |             |
|           |        |     |                  | g.44905697                  | na              | Pathogenic | na/na/dc | 0.0000083 (0.0000083)     | rs312262752          |            |             |
| SPG0702   | SPGII  | 15  | g.44943869       | NM_025137.3:c.1275insA      | c_ht            | na/na/dc    | Pathogenic | na/na/dc                 | 0                      | na         | AR          |
|           |        |     |                  | NP_079413.3:p.(Glu246Argfs*3) |                |              |          |                          |                        |            |             |
|           |        |     |                  | c.(4906 + 1_4907–1)del      |                |              |          |                          |                        |            |             |
|           |        |     |                  | (deletion of exon 29)       |                |              |          |                          |                        |            |             |
|           |        |     |                  | g.44876255                  | 0.705           | Pathogenic | na/na/dc | 0.000006 (0.000041)       | rs141848292          |            |             |
| SPG0502   | SPGII  | 15  | g.44941211       | NM_025137.3:c.1457-2A>G     | c_ht            | 3652         | Pathogenic | na/na/dc                 | 0                      | rs312262726 | AR          |
|           |        |     |                  | (splice acceptor variant)   |                |              |          |                          |                        |            |             |
|           |        |     |                  | NP_079413.3:p.(Leu950Trpfs*13) |                |              |          |                          |                        |            |             |
|           |        |     |                  | c.(1735 + 1_1736–1)del      |                |              |          |                          |                        |            |             |
|           |        |     |                  | (deletion of exons 9–11§)   |                |              |          |                          |                        |            |             |

§ Deletion of exons 29 and 30.
respectively. Two probands had pure HSP, while in one with the nonsense variant, a complicated HSP phenotype with neuropathy as an additional symptom was observed.

**WASHC5 (SPG8)**

The WASHC5 missense variants were found in two HSP probands and at least one affected individual within their families. Patient SPG0302 was found to have WASHC5 c.1859C>T (p.Val620Ala). The female proband and her affected sibling—aged 39 and 37 years at onset—had frontal cortex atrophy. Moreover, in patient SPG0302, white matter and thoracic spinal cord lesions were present. The male proband SPG0403, with WASHC5 c.647C>T (p.Pro216Leu), presented a complex HSP with dysarthria. His brother with the same variant had intellectual disability in addition to HSP (but he had a verified birth asphyxia—a possible cause of the brain damage).

**KIF5A (SPG10)**

Two KIF5A variants were identified in two probands. One of them, KIF5A c.484C>T (p.Arg162Trp), which localised in motor domain of the kinesin protein was present in a proband with pure HSP and onset of symptoms at age 41. The second, KIF5A variant c.1402C>T (p.Arg468Trp), which altered the stalk part of the protein, was identified in a female proband with pyramidal signs, ataxia, dysdiachokinesia, bradykinesia, titubation, ophthalmoparesis and dementia, in whom first symptoms appeared after turning 40. In MRI, marked atrophy of the cerebellum and cerebral cortex (predominantly temporal and parietal) was observed.

**KIF1A (SPG30)**

A heterozygous KIF1A c.962G>A (p.Gly321Asp) variant, localised in the motor domain of the protein, was found in an AD pedigree. The female proband and her mother had childhood onset, complex hereditary spastic paraplegia and cognitive decline.

**Autosomal recessive HSPs**

**SPG11 (SPG11)**

The NGS analysis enabled us to identify ten different SPG11 variants (with the ExAC frequency below 0.005) in seven probands. In all of them, the variants were present in both alleles. In the SPG1002 proband, three different variants were detected. In three other patients with single variants found in this study, SPG0103, SPG0301 and SPG0702, the microrearrangements: duplication of exons 28–29, deletions of exons 9–11 and exon 29, respectively, were localised in trans. Five of the variants were frameshift deletions or
| Patient ID | Gene | Chr Genomic position | cDNA change | Allele zygocity | PhyloP score | ClinVar SIFT/PolyPhen/MutTaster | ExAC allele frequency† | rs number | Inheritance |
|------------|------|----------------------|-------------|-----------------|-------------|-------------------------------|-----------------------|------------|-------------|
| SPG0403    | WASHC5 | 8 | g.126091044 | NM_014846.3:c.647C>T | ht | 5443 | na tol/pb_dam/dc | 0.001694 (0.00122) | rs72720524 | AD          |
| SPG1402    | KIF5A | 12 | g.57965883 | NM_004984.2:c.1402C>T | ht | 1.19 | na del/bn/dc | 0 (0.0000084) | rs771021589 | AD          |
| SPG0303    | ITPR1 | 3 | g.4716885 | NM_001168272.1:c.2687C>T | ht | 1719 | us tol/bn/dc | 0.00051 (0.000315) | rs201519806 | AD          |
| SPG0401    | ITPR1 | 3 | g.4716885 | NM_001168272.1:c.2687C>T | ht | 1719 | us tol/bn/dc | 0.00051 (0.000315) | rs201519806 | AD          |
| SPG1203    | ITPR1 | 3 | g.4725441 | NM_001168272.1:c.3412A>G | na | 4274 | us tol/bn/dc | 0.0008452 (0.000484) | rs199698357 | AD          |
|            |       | 3 | g.4821291 | NM_001168272.1:c.6304G>T | NA  | 4331 | del/bn/dc | 0.000105 (0.000058) | rs373973399 |             |
| SPG0503    | SETX | 9 | g.135140243 | NM_015046.5:c.7417C>G | ht | 3436 | na tol/ps_dam/pol | 0 (0.000033) | rs760196991 | AD          |

† according European (non-Finnish) population, total frequency in bracket
bn benign, dc disease causing, del deleterious, ht heterozygous, na not applicable, pb_dam probably damaging, pol polymorphism, ps_dam possibly damaging, rs reference SNP, tol tolerated, us uncertain significance
insertions, two were in-frame deletions, one was in the splice-site, one was nonsense and one was a missense change. In SPG1002, the missense variant was identified in cis with the frameshift one.

All of the seven SPG11 probands had a complicated form of HSP and showed cognitive impairment: dysarthria 5/7; dysphagia 2/7; nystagmus 3/7; ophthalmoparesis (horizontal gaze) 2/7; cervical dystonia 1/7 and mild ataxia 3/7. In neuro-imaging performed in six probands, thin corpus callosum was found in 5/6, periventricular white matter lesions were found in 4/6, and mild cortical and subcortical atrophy was identified in 2/6. EMG provided evidence of polyneuropathy in three out of five examined probands.

**CYP27A1 (CTX)**

In one proband, NGS revealed a homozygous variant, c.379C>T (p.Arg127Trp) in the CYP27A1 gene, known as pathogenic in cerebrotendinous xanthomatosis (CTX). The carrier status (heterozygosity) was confirmed in the proband’s father. The patient, with pyramidal and cerebellar signs, petit mal seizures, bilateral cataract and retinal degeneration in the right eye, was classified as a case of the complicated HSP. Mild cortical and subcortical atrophy were present in brain MRI. Furthermore, in the patient’s medical history, vitamin B12 deficiency and nephrolithiasis were documented. To date, neither xanthomas nor other signs characteristic for CTX were not observed in the patient.

**Genes with uncertain significance in HSPs**

**ITPR1** (GLSP/SCA15/SCA29)

Three different variants of uncertain significance were identified in the ADHSP patients. **ITPR1**: c.2687C>T (p.Ala896Val) was identified in seven individuals from two unrelated families with pure HSP. In the SPG1203 proband, two different ITPR1 variants (c.3412A>G-p.Met1138Val and c.6304G>T-p.Ala2102Ser) were found. A female patient with weakness and spasticity of her lower limbs, balance disturbances and polyneuropathy had onset of symptoms at age 50. Genetic testing in her relatives was impossible; however, her family history may indicate AD inheritance. All the pedigrees and localization of identified ITPR1 variants are shown in Fig. 2.

**SETX** (ALS4/SCAR1)

One **SETX** missense variant of uncertain significance, c.7417C>G (p.Leu2473Val), was detected in a 2-year-old proband and the father, who has been affected since childhood. The father’s neurological examination showed upper and lower limb weakness and spasticity with increased tendon reflexes and clonus.

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**Discussion**

Due to heterogeneity, the increasing number of involved genes and varieties of phenotypes (disorders) linked to a single gene, the classification and diagnostics of HSPs are challenging. To overcome these difficulties, different NGS approaches have been applied in a number of studies, mostly targeted sequencing but also whole exome sequencing [13–17]. In the present study, we analysed 30 HSP index cases using the Illumina TruSightTM One NGS sequencing panel. Bioinfomatic analysis was performed for 132 out of the 4813 genes included in the panel. This methodology allowed us to identify 25 variants in nine genes. The pathogenic and likely pathogenic variants were identified in 16 probands. In five of them, in whom only MLPA technique had been used for microrearrangement searching, we identified three **SPAST** and two **ATL1** variants by NGS. It is an evidence that MLPA is not sufficient for SPG4 testing alone, nonetheless together with NGS is now a standard in diagnostic approach. Less frequent HSP subtypes were identified in a group of patients in whom the **SPAST**, **ATL1** and **REEP1** pathogenic variants had been previously excluded. Two different variants were identified in **WASHC5** (SPG8, OMIM #603563, previously known as **KIAA0196**) and **KIF5A** (SPG10, OMIM #604187) genes, both regarded as rare HSP subtypes (approximate frequency 1–2%) that may be associated with pure or complicated HSP phenotypes [4]. The **WASHC5**: c.1859T>C (p.Val620Ala) variant has previously been detected in pure HSP patients but has not been reported in either ExAC or the 1000 Genomes projects [18]. The **KIF5A**: c.484C>T (p.Arg162Trp) variant has been reported in a three-generation pedigree with spastic paraplegia as a primary symptom [19].

**KIF1A** is a neuron-specific motor protein involved in intra-cellular transport along microtubules. Variants in the **KIF1A** gene have been described in patients with AR hereditary sensory and autonomic neuropathy type 2 (HSAN2, OMIM #614213) and subtype 30 of the hereditary spastic paraplegia (SPG30, OMIM #610357) [20–23]. De novo **KIF1A** variants with AD transmission have been identified in multiple cases with childhood onset of intellectual disability and a number of neurological signs, such as progressive spastic paraplegia, optic nerve atrophy, peripheral neuropathy and cerebral and/or cerebellar atrophy, have been variously classified as autosomal dominant mental retardation type 9 (MRD9, OMIM#614255) [24–28] or complicated hereditary spastic paraplegia [25, 29, 30]. Finally, **KIF1A** mutations have been found in pure HSP subjects [30–32]. In the present study, a dominant **KIF1A** variant localised in the motor domain of the protein was found in a female proband and her mother with childhood onset complex HSP and cognitive decline. Twenty-three out of 25 heterozygous **KIF1A** variants (including the present study) alter the highly conserved motor domain of the protein. However, two out of four variants responsible for recessive HSP and any of the variants identified in HSAN2 are localised in the motor
This suggests that localization of the KIF1A variants within the gene is not adequate evidence for phenotype transmission. Moreover, the latest data indicate that dominant conditions, including ADHSP, linked with KIF1A variants are more frequent than recessive ones.

SPG11 (OMIM #604360) is the only known recessive HSP subtype identified in this study. Contrary to other studies, we have not detected any affected patient with CYP7B1 (SPG5, OMIM #270800) or SPG7 (SPG7, OMIM #607259) mutations, or any mutation carriers [13–17, 32]. Moreover, variants in ZFYVE26 (SPG15, OMIM #270700), which occur with frequency below 0.005 in the ExAC database, were not detected in our cohort.

In addition to the recessive variants, in one case, we detected a homozygous variant in the CYP27A1 gene. Pathogenic variants in the cytochrome P450 CYP27A1 gene result in the production of a defective sterol 27-hydrolase enzyme and have been linked with cerebrotendinous xanthomatosis (CTX) (OMIM #213700). Clinical manifestation of CTX includes neurological dysfunction (e.g. cerebellar ataxia, pyramidal signs, and seizures), cataracts, tendon xanthomas and chronic diarrhea [33, 34]. However, some atypical presentation of symptoms may occur. For example, Verrips et al. described seven patients with CYP27A1 variants and slowly progressive spinal cord syndrome classified as spinal xanthomatosis. Moreover, similar to our case, all of the patients presented pyramidal signs, and in five of them, spinal cord white matter lesion have been demonstrated. Six out of seven cases studied by Verrips et al. did not have tendon xanthomas [35]. Patients with CYP27A1 variants affected with pure and complicated HSP but without xanthomas were also described by Burguez et al. and Nicholls et al. [15, 36]. These findings suggest that patients with CYP27A1 variants may
present the broader clinical spectrum including HSP phenotype, nonetheless the lack of the typical symptoms of CTX, especially xantomas, should not exclude the investigation of CYP27A1 gene mutations.

Variants of uncertain significance within ITPR1 and SETX genes were detected in four cases. ITPR1 variants have already been described as possibly corresponding to four different phenotypes: multi-exon deletions in ITPR1 gene to spinocerebellar ataxia type 15 (SCA15, OMIM #606658), single nucleotide variants to spinocerebellar ataxia type 29 (SCA29, OMIM #117360) or ataxic cerebral palsy (Ataxic CP), and the truncated and splice-site variants in Gillespie Syndrome (GLSP, OMIM #206700) also presented ataxia and balance disturbances [37–42]. ITPR1 encodes a homotetramer calcium channel protein that modulates intracellular calcium signalling. Its primary structure consists of three major domains [43]. In this study the ITPR1 c.2687C>T (p.Ala896Val) variant was detected in two unrelated families and segregates with pure HSP phenotype in seven cases. We also identified two different ITPR1 variants in a patient with pyramidal signs and polyneuropathy. Although the three described variants were reported in the ExAC database, their frequency was lower than 0.005 (Table 2b). The relatively mild HSP symptoms in our patients were first observed in adulthood i.e. the age of onset was not optimal for control studies. The segregation data in the families with c.2687C>T (p.Ala896Val) supports its pathogenicity; however, according to the ACMGG&AMP guidelines, this is not adequate evidence to classify it as a pathogenic/probably pathogenic variant. Variants identified in the present study are localised in the coupling-domain and comprise the first report assigning ITPR1 variants to HSP.

A variant classified as of uncertain significance was also found in the senataxin gene. SETX variants are responsible for AR spinocerebellar ataxia (SCAR1) and AD amyotrophic lateral sclerosis (ALS4) [44–48]. The heterozygous variant of the SETX gene has also been described as a cause of hereditary motor neuropathy (dHMN) [49, 50]. Taniguchi et al. reported a family with a SETX variant misdiagnosed as a hereditary spastic paraplegia [51]. The mentioned variant (SETX c.8C>T) was localised in the N-terminal end of the protein, different than the SETX c.7417C>G (p.Leu2473Val), altering the C-terminal part of the protein, which was identified during our study in father and son with pure HSP. It is localised in the region of the helicase domain, where known pathogenic variants correlated with ALS4 and SCAR1 phenotypes had been reported as well [52].

Although the molecular investigation of rare heterogeneous disorders, such as hereditary spastic paraplegias, will soon be based on massive NGS technology, their molecular aetiology assessment still remains challenging. Two major difficulties to face at present are: (1) interpretation of the detected variants (pathogenic vs benign) and (2) classification of the identified variant and its association with a specific disease. Unified and reliable sequence variants interpretation guidelines were developed by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Each rare or novel variant should be evaluated in a patient’s and family’s history context, and physical examination and previous differential diagnosis should be performed. Such clinical evaluation is supportive during the process of variants classification as disease-causing, incidental or benign findings [12]. Variants classified as pathogenic but also likely pathogenic have sufficient evidence to be used in genetic counselling and clinical decision-making. In contrast, variants of uncertain significance need further investigation that may result in their reclassification [12].

Implementing NGS technologies in clinical practice also brings problems due to the genotype-phenotype correlation and variants’ classification. The classification systems were designed according to a predominant disease phenotype and/or a mode of inheritance. Currently, various genes corresponding to numerous complex phenotypes, such as spinocerebellar ataxias, spastic paraplegias and amyotrophic lateral sclerosis, are associated with SPG7, SPG11, PNPLA6, KIF1C and SETX, and they may be inherited as both autosomal dominant and recessive traits (KIF1A, REEP2, AFG3L2, SETX). In clinical practice, it becomes problematic whether the identified gene variant should be classified as corresponding to a new phenotype or if it “fits” the patient’s genotype consistent with the previous clinical diagnosis. Synofzik et al. proposed introducing the unbiased modular phenotyping approach to replace the ataxias and hereditary spastic paraplegia classification [6]. In parallel, we also recommend simultaneously testing and analysing the HSP, SCA and ALS genes due to their overlapping phenotype and common cellular pathways involved.

In this paper, we report 24 different variants of nine genes in HSP patients. Seven of the variants are novel. They were classified according to the ACMGG&AMP guidelines, and nine were classified as pathogenic, nine as likely pathogenic and six as of uncertain significance. Among nine analysed genes, five have already been known as directly associated with HSP. NGS testing revealed genetic variants in 22 out of 30 tested families. Altogether with the previous study [8], seven different HSP subtypes have been diagnosed in the Polish group of patients to date. Our data also support the evidence that KIF1A (SPG30) variants are more frequent in patients with ADHSP, although they were primarily identified as ARHSP. Moreover, we believe that CYP27A1 variants should be considered to be complicated HSP phenotype cases, as well.

The overlapping phenotypes of HSP, SCA and ALS are associated with multiple genes; therefore, NGS-based screening provides the best comprehensive genetic diagnostic approach. The most challenging interpretation of the novel variants requires the entire body of clinical and molecular evidence available in the entire studied group of patients sharing a defined spectrum of clinical signs.
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