Application of a custom NGS gene panel revealed a high diagnostic utility for molecular testing of hereditary ataxias

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Abstract
Hereditary ataxias (HA) are a rare group of heterogeneous disorders. Here, we present the results of molecular testing of a group of ataxia patients using a custom-designed next-generation sequencing (NGS) panel. Due to the genetic and clinical overlapping of hereditary ataxias and spastic paraplegias (HSP), the panel encompasses together HA and HSP genes. The NGS libraries, comprising coding sequences for 152 genes, were performed using KAPA HyperPlus and HyperCap Target Enrichment Kit, sequenced on the MiSeq instrument. The results were analyzed using the BaseSpace Variant Interpreter and Integrative Genomics Viewer. All pathogenic and likely pathogenic variants were confirmed using Sanger sequencing. A total of 29 patients with hereditary ataxias were enrolled in the NGS testing, and 16 patients had a confirmed molecular diagnosis with diagnostic accuracy rate of 55.2%. Pathogenic or likely pathogenic mutations were identified in 10 different genes: POLG (PEOA1, n = 3; SCAE, n = 2), CACNA1A (EA2, n = 2), SACS (ARSACS, n = 2), SLC33A1 (SPG42, n = 2), STUB1 (SCA48, n = 1), SPTBN2 (SCA5, n = 1), TGM6 (SCA35, n = 1), SETX (AOA2, n = 1), ANO10 (SCAR10, n = 1), and SPAST (SPG4, n = 1). We demonstrated that an approach based on the targeted use of the NGS panel can be highly effective and a useful tool in the molecular diagnosis of ataxia patients. Furthermore, we highlight the fact that a sequencing panel targeting both ataxias and HSP genes increases the diagnostic success level.

Keywords Next-generation sequencing · Neurodegenerative diseases · Hereditary ataxias · Hereditary spastic paraplegias · Rare genetic variants

Introduction
Hereditary ataxias (HAs) are a group of complex and progressive neurodegenerative disorders that present various modes of inheritance. Accordingly, they can be classified as spinocerebellar ataxias (SCA – AD), episodic ataxias (EA – AD), autosomal recessive spinocerebellar ataxias (SCAR), and spastic ataxias (SPAX – AD/AR). To date, 48 genes have been associated with SCA, 61 with SCAR, 9 with EA, and 6 with SPAX. In addition, seven genes are known to be involved in X-linked cerebellar ataxias, and 4 are involved in ataxias with mitochondrial disorders (Bird 2019). Despite the fact that many ataxia genes have already been discovered, novel candidate genes are still emerging (Valence et al. 2019).

Clinically, the phenotype may include variable features, but generally, the disease is characterized by progressive cerebellar syndrome with balance and coordination problems, gait and limb ataxia, hypotonia, clumsiness, or speech and eye movement abnormalities, whereas the latter can display early or young adult onset of the disease. Moreover, some patients can present with overlapping features, and there may be intrafamilial/interfamilial phenotypic variability (including different ages at onset and severity of symptoms) or incomplete penetrance (Angelini et al. 2019). Additionally, mutations in the same SCA genes may lead to distinct phenotypes or exhibit different modes of inheritance. Cerebellar
ataxia is a neuropathological and neuroimaging hallmark of ataxia.

Genetically, the molecular background of ataxias varies widely, ranging from point and small mutations to dynamic ones. The latter account for 45% of all autosomal dominant cerebellar ataxia cases (Durr 2010). Therefore, due to this clinical and genetic heterogeneity, the establishment of an ataxia diagnosis may be very complicated and challenging. Recent studies have shown that many patients remain undiagnosed after screening for the most common repeat expansions (Brusco et al. 2004). The implementation of next-generation sequencing (NGS) approaches, including whole-genome sequencing (WGS), whole-exome sequencing (WES), and targeted gene panel sequencing (TGP), enables comprehensive screening of many genes simultaneously. This can greatly increase the possibility of identifying the genetic cause of these conditions. Currently, custom gene testing is one of the most widely used diagnostic tools for heterogeneous neurodegenerative disorders in clinical practice.

In this study, we present the results of a genetic analysis using a custom-designed panel encompassing all known genes for hereditary ataxia and hereditary spastic paraplegia (HSP) in a cohort of Polish patients. Furthermore, we assessed the efficiency of our NGS panel as a diagnostic tool for the possible expansion of the phenotypic and genotypic spectrum of ataxias in clinical practice.

Materials and methods

This study was approved by the Ethics Commission of IPiN. All individuals participating in the research gave their written informed consent.

Participants

In total, 29 patients fulfilling the following criteria were included: cerebellar gait and/or limb ataxia, and exclusion of the most common nucleotide repeat expansion loci, SCA1, SCA2, SCA3, SCA6, SCA7, SCA8, SCA17, SCA36, and dentatorubral-pallidoluysian atrophy (DRPLA). Acquired cases of ataxia were excluded. Our tested cohort involved 15 sporadic cases (51.7%), 9 familial cases (31.0%) and 5 with unknown family history (17.2%). Most of the patients displayed incoordination and unsteadiness, dysarthria, dysdiadochokinesia, extensor plantar responses, increased deep tendon reflexes, spasticity, neuropathy, nystagmus, pes cavus, and dysmetria. Other neurological features included action tremor, hypertonia, epilepsy, cognitive impairment, and pyramidal tract dysfunction. To assess the severity of ataxia, the Scale for the Assessment and Rating of Ataxia (SARA) was applied. The following additional examinations were performed on particular individuals: brain magnetic resonance imaging (MRI, 15/29), electromyography (EMG, 9/29), computed tomography of the spine (CT, 5/29), electrocardiography (ECG/EKG, 5/29), electrophysiology (ENG, 4/29), head CT (4/29), electroencephalography (EEG, 4/29), cerebrospinal fluid (CSF) testing (3/29), nerve conduction velocity (2/29), chest X-ray (2/29), visual evoked potential (VEP, 1/29), Doppler ultrasonography (1/29), ultrasonic imaging of the abdomen (1/29), and skin and skeletal muscle biopsy (1/29).

Methods

Genomic DNA was extracted from the peripheral blood using the MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume (Roche), following the manufacturer’s instructions. The quantity and quality of the isolated DNA were assessed with a UV/VIS Spectrophotometer Nanodrop2000 (Thermo Fisher Scientific) and Qubit fluorometer (Invitrogen, Thermo Fisher Scientific).

For the present study, a targeted NGS gene panel comprising coding sequences for 152 known genes associated with hereditary ataxias (88) and hereditary spastic paraplegias (64) was developed. The list of included genes was based on data in GeneReviews, scientific literature, and the following databases: Orphanet (https://www.orpha.net/consor/cgi-bin/index.php?lng=EN), OMIM (https://www.omim.org/), and GeneCards (https://www.genecards.org/) (alphabetical list – Supplementary table 1).

The library of patient DNA was prepared from 250 ng genomic DNA with a KAPA HyperPlus Kit (Roche), according to the manufacturer’s instructions. The protocol included the following steps: library preparation, hybridization, bead capture, washing, amplification enrichment QC, sequencing, and pre- and post-capture multiplexing. Quantification analysis and assessment of the average size and length of the NGS libraries were performed using a Bioanalyzer assay (Agilent).

Sequencing on the NGS libraries was performed by a MiSeq (Illumina) paired-end 2×75-bp DNA sequencing platform with a MiSeq Reagent Kit v3 (150-cycle, Illumina), according to the manufacturer’s procedure.

The analysis of gene variants was performed with the use of the BaseSpace Variant Interpreter, and interpretation was performed according to the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) Standards and Guidelines (Richards et al. 2015). The initial variation filtering included the following: (1) all coding consequences (stop gain or loss,
splice site, indels, missense, and protein altering); (2) GnomAD frequency value less than 2% for all populations; and (3) small variant QC metrics with value >25% for variant read frequency. To investigate the functional predictions of the variants by several in silico online programs, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (https://sift.bii.a-star.edu.sg/), and MutationTaster (http://www.mutationtaster.org/) were used. The analysis and interpretation of the clinical significance of the DNA variants were determined by using ClinVar, the Leiden Open Variation Database (LOVD), and the Human Gene Mutation Database (HGMD), and the variant frequency in populations was assessed by Genome Aggregation Database (gnomAD). The assessment of the quality of the NGS data was performed by using the genome visualization tool Integrative Genomics Viewer (IGV) and included only variants that had ≥15 reads.

Sanger sequencing

All pathogenic or likely pathogenic variants identified by NGS were confirmed using Sanger sequencing on an ABI 3130 genetic analyzer. The available additional (affected and healthy) family members were tested for the segregation of identified pathogenic/likely pathogenic variants and variants of uncertain significance (VUS).

Results

General characteristics of the patients

The 29 patients enrolled in the study presented progressive ataxia as the main clinical manifestation. Adult onset was observed in 48.3% of cases (14/29), whereas the remaining 37.9% (11/29) were young adult onset (18–29 years) and 13.8% (4/29) were childhood onset (≤18 years). The mean and median age of the study participants at disease onset was 32.7 (range between 7 and 70) and 26.0 years, respectively. The cohort comprised 14 male (48.3%) and 15 female individuals (51.7%).

In total, for 16 (55.2%) out of 29 patients, we were able to make a definite molecular diagnosis (Table 1). Within the 16 probands we diagnosed, the following were given: progressive external ophthalmoplegia with mitochondrial DNA deletions type 1 (PEOA1) (n = 3), spinocerebellar ataxia with epilepsy (SCAE) (n = 2), episodic ataxia type 2 (EA2) (n = 2), spastic ataxia of Charlevoix-Saguenay (ARSACS) (n = 2), spastic paraplegia type 42 (SPG42) (n = 2), spinocerebellar ataxia type 48 (SCA48) (n = 1), spinocerebellar ataxia type 35 (SCA35) (n = 1), spinocerebellar ataxia type 5 (SCA5) (n = 1), ataxia-oculomotor apraxia type 2 (AOA2) (n = 1), spinocerebellar ataxia type 10 (SCAR10) (n = 1), and spastic paraplegia type 4 (SPG4) (n = 1). For one of the patients, the genetic background is still under debate because of the coexistence of pathogenic variants in SPAST and POLG genes that are associated with different phenotypes. SPAST is associated with spastic paraplegia type 4, and POLG is associated with progressive external ophthalmoplegia with mitochondrial DNA deletions type 1. Moreover, the number of years from disease onset to diagnosis ranged from 5 to 28, with an average diagnostic delay of 13.9 years.

NGS parameters

The mean coverage depth of each region was estimated to be 110.0x, with the highest score of 153.4x and the lowest of 68.1x. The mean proportion of the sequence achieving 30x sequencing coverage was 95.5% and ranged from 89.6 to 97.2%.

Pathogenic/likely pathogenic variants

In total, 20 putative pathogenic or likely pathogenic mutations were identified in the following genes: POLG, CACNA1A, SACS, SLC33A1, STUB1, SPTBN2, TGM6, SETX, ANO10, and SPAST. Of these, 5 were already known, and 15 were novel. Pathogenic variants in POLG, CACNA1A, SACS, and SLC33A1 accounted altogether for 68.8% of the variants (Table 2). Pathogenic variants in SETX, STUB1, SPTBN2, ANO10, SPAST, and TGM6 constituted 37.5% of variants and were found in single cases.

The detected mutations included missense (13/20; 65.0%), frameshift (4/20; 20.0%), stop-gain (2/20; 10.0%), and splice-site mutations (1/20; 5.0%).

One of the patients presented an ultrarare variant in the ANO10 gene due to consanguinity. A homozygous splice site pathogenic mutation in ANO10, c.1218+1G>C, was identified in a 33-year-old man (patient HA). Sanger sequencing was performed for the affected proband and both asymptomatic parents, who were heterozygous carriers for this mutation (Fig. 1). The affected patient clinically presented with gait and limb ataxia, dysarthria, intention tremor, and age of onset of 20 years. Remarkably, he also manifested other neurological conditions, such as increased deep tendon reflexes, extensor plantar responses, dysdiadochokinesia, and spasticity. Brain MRI showed bilateral cortical atrophy and cerebellar vermis.

Variants of uncertain significance

In the remaining patients (13/29), more than one variant of uncertain significance (VUS) per case was identified. Interpretation of these variants is difficult and requires
| Case | Clinical features | Neuroimaging | Gene (transcript) | Mutation | SIFT Polyphen-2 MutationTaster | Mutations known or novel | Diagnosis (mode of inheritance) |
|------|-------------------|--------------|------------------|----------|-------------------------------|--------------------------|-----------------------------|
| HK   | Ataxia and unsteadiness, episodic; Dysarthria, myoclonic epilepsy | Normal MRI (EEG with paroxysmal activity) | CACNA1A (NM_023035.2) | c.2042_2043delAG; (p.Gln681ArgfsTer103); het | NA | NA (Disease causing) | Known^2 EA2 (AD) |
| RC   | Ataxia and unsteadiness, episodic; Vertigo, dysarthria, migraine headache, epilepsy, saccadic smooth pursuit, axonal sensorimotor polyneuropathy | Atrophy of cerebellar vermis (EEG with paroxysmal activity) | CACNA1A (NM_023035.2) | c.859T>C; (p.Cys287Arg); het | Deleterious (Probably damaging) | Disease causing | Novel EA2 (AD) |
| ST   | Ataxia, dysarthria, pyramidal tract dysfunction, cerebellar ataxia, cognitive impairment, optic atrophy | Atrophy of the superior vermis and cerebellar hemispheres | POLG (NM_002693.2) | c.2615G>A; (p.Ser872Asn); het | Deleterious (Probably damaging) | Disease causing | Novel PEOA1 (AD) |
| RM   | Gait and limb ataxia (lower limbs more affected than upper limbs), positive Romberg sign, motor and sensory neuropathy, decreased sensory nerve conduction velocity, impaired vibration sense in the right foot, cardiomyopathy | Normal MRI | POLG (NM_002693.2) | c.1399G>A; (p.Ala467Thr); het | Deleterious (Benign) | Disease causing | Known^3 PEOA1 (AD) |
| KK   | Ataxia, gait ataxia, dysarthria, slurred speech | Cerebellar atrophy | STUB1 (NM_005861.3) | c.146A>G; (p.Tyr49Cys); het | Deleterious (Benign) | Disease causing | Novel SCA48 (AD) |
| LM   | Difficulty walking, spastic paraplegia, hypertonia | Nd | TGM6 (NM_198994.2) | c.632G>A; (p.Arg211His); het | Deleterious (Benign) | Disease causing | Novel SCA35 (AD) |
| CM   | Cerebellar ataxia (lower limbs more affected), tremor, dysarthria, positive Romberg sign | Atrophy of the superior vermis and cerebellar hemispheres | SPTBN2 (NM_006946.2) | c.3824G>A; (p.Arg1275Gln); het | Tolerated (Possibly damaging) | Disease causing | Novel SCA5 (AD) |
| KW   | Spastic gait (walk on crutches), hyperreflexia, extensor plantar responses, pes cavus | Normal MRI | SPAST (NM_014946.3) | c.1506delA; (p.Lys502AsnfsTer28); het | NA | NA (Disease causing) | Novel SPG4 (AD) |
| SA   | Cerebellar and pyramidal syndrome, peripheral neuropathy | Cerebellar atrophy | SLC3A1 (NM_004733.3) | c.1559T>C; (p.Ile520Thr); het | Deleterious (Benign) | Disease causing | Novel SPG42 (AD) |
Table 1 (continued)

| Case | Clinical features | Neuroimaging | Gene (transcript) | Mutation | SIFT Polyphen-2 MutationTaster | Mutations known or novel | Diagnosis (mode of inheritance) |
|------|-------------------|--------------|------------------|----------|--------------------------------|--------------------------|-----------------------------|
| BJ   | Ataxia, cerebellar syndrome, impaired tandem gait, unstable heel-to-toe walk, dystaxia, intention tremor (mild), dysdiadochokinesia (delay), scanning speech, dysarthria, vertigo, decreased muscle tone and tendon reflex of upper and lower limbs, nystagmus, cloudy vision, changes in writing, Insulin-dependent diabetes, hypercholesterolemia | Marked cortical and subcortical atrophy of cerebellum with symmetrical atrophy for both hemispheres of the cerebellum and cerebellar vermis | SLC33A1 (NM_004733.3) | c.1559T>C; (p.Ile520Thr); het | Deleterious | Possibly damaging | Disease causing | Novel | SPG42 (AD) |
| AL   | Gait ataxia, delayed walking development, increased falls, cerebellar ataxia, deep tendon reflexes, pes cavus | Atrophy of the superior vermis and cerebellar hemispheres, linear hypointensities in the pons | SACS (NM_014363.5) | c.5773_5779delCTGAGTG; (p.Leu1925SerfsTer11); het c.11374C>T; (p.Arg3792Ter); het | Both NA | Both NA | Both disease causing | Novel | ARSACS |
| KM   | Nd | Nd | SACS (NM_014363.5) | c.8340delT; (p.His2781MetfsTer4); het c.7960T>G; (p.Tyr2654Asp); het c.5498C>T; (p.Ser1833Phe); het | NA | NA | Disease causing | Three novel | ARSACS |
| KS   | Gait and limb ataxia, dysdiadochokinesia (mild), dysarthria, dysphagia, fasciculations, myoclonus, hypertonia, migraine, epilepsy, deep tendon reflexes, cognitive impairment, extensor plantar responses, hearing loss (mild), facial asymmetry (mild), spinal deformity: lordosis, kyphosis, scoliosis, ankle and patellar clonus, bilateral thalamic lesions | Brain atrophy (mainly frontal and temporal lobe) and atrophy of cerebellum in MRI (EEG with abnormal activity) | POLG (NM_002693.2) | c.428C>T; (p.Ala143Val); het c.2323G>A; (p.Glu775Lys); het | Both deleterious | Both disease causing | Known | SCAE (AR) |

Autosomal recessive
| Case | Clinical features | Neuroimaging | Gene (transcript) | Mutation | SIFT Polyphen-2 MutationTaster | Mutations known or novel | Diagnosis (mode of inheritance) |
|------|-------------------|--------------|------------------|----------|-------------------------------|--------------------------|-------------------------------|
| IJ   | Gait and limb ataxia, dysarthria, vertigo, positive Romberg sign, hearing loss, nystagmus, upward gaze paresis, ophthalmoparesis, intestinal pseudo-obstruction, motor-sensory axonal neuropathy | Vasogenic damage in cerebellum without brain and cerebellum atrophy (EMG—axonal damage) | POLG (NM_002693.2) | c.2243G>C; (p.Trp748Ser); hom | Deleterious | Disease causing | Known<sup>11</sup> | SCAE (AR) |
| SJ   | Nd                | Nd           | SETX (NM_015046.5) | c.7365_7366delTA; (p.Tyr2455Ter); het c.7252A>G; (p.Ser2418Gly); het | NA | Disease causing | Deleterious Possibly damaging | Disease causing | Two novel | AOA2 (AR) |
| HA   | Gait and limb ataxia, dysarthria, increased deep tendon reflexes, extensor plantar responses, intention tremor, dysdiadochokinesia, spasticity | Bilateral cortical and cerebellar vermis atrophy | ANO10 (NM_001346464.1) | c.1218+1G>C; hom | NA | Disease causing | NA | SCAR10 |

The damaging predictions on nonsense, splicing, and frameshift mutations are not applicable by using in silico prediction bioinformatic tools, e.g., SIFT and Polyphen-2.

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; MRI, magnetic resonance imaging; het, heterozygous; hom, homozygous; Nd, not determined; NA, not applicable; EA2, episodic ataxia type 2; PEOA1, progressive external ophthalmoplegia with mitochondrial DNA deletions type 1, autosomal dominant; SCA48, spinocerebellar ataxia type 48; SCASAL, spinocerebellar ataxia type 35; SCA5, spinocerebellar ataxia type 5; SPG4, spastic paraplegia type 4, autosomal dominant; SPG42, spastic paraplegia type 42, autosomal dominant; ARSACS, autosomal recessive spastic ataxia of Charlevoix-Saguenay; SCAE, spinocerebellar ataxia with epilepsy; AOA2, ataxia-oculomotor apraxia type 2; SCAR10, spinocerebellar ataxia type 10.
a more detailed analysis, including clinical and familial interpretation, for the proper identification of the most likelihood disease-causing mutations (Table 3). Among them, 2 patients had a VUS with a dominant mode of inheritance (AFG3L2, CCDC88C), and 3 had a single VUS in a recessive manner (CAPN1, SACS, CLCN2). In 5 individuals, the interpretation of genetic findings remained unknown because of the complexity of genotype–phenotype correlation, and in 3 cases, it ultimately remained undiagnosed.
| Case | Clinical features | Imaging | Family history | Gene (transcript) | Mutation | Comments |
|------|------------------|---------|----------------|------------------|----------|----------|
| WS   | 4 limbs ataxia, gait ataxia, tandem gait, dystarhria, dystonia (torticollis, blepharospasm, laryngeal dystonia) | Cerebellar atrophy (hemispheres and vermis) | Familial | AFG3L2 (NM_006796.2) | c.2062C>G; (p.Pro688Ala); het | Lack of sample DNA of other family members for segregation analysis. Functional tests required to confirm pathogenicity. |
| BK   | Spastic paraparesis, tremor of fingers, patellar and ankle clonus, myopathy, deficits of memory, high palatal vault | Nd | Familial | CCDC88C (NM_001080414.3) | c.5087T>C; (p.Leu1696Pro); het | Additional likely pathogenic heterozygous mutation c.660C>A (p.Tyr220Ile) was detected in the ACTA1 gene (included in other NGS panel). |
| PI   | Gait ataxia, involuntary movements, clumsiness, dyslexia, slurred speech, dysmetria (mild), action tremor, strabismus, axonal peripheral polyneuropathy, increased alpha-fetoprotein | Cerebellar atrophy | Sporadic | CAPN1 (NM_001198868.1) | c.1474G>A; (p.Gly492Arg); het | Autosomal recessive inheritance, no second mutation found |
| ZBD  | 4 limbs ataxia, tremor, gait ataxia, dystarhria, pyramidal signs | Generalized small cortico-subcortical brain atrophy | Sporadic | SACS (NM_014363.5) | c.3408T>G; (p.Asn1136Lys); het | Additional likely benign heterozygous mutation c.171+6C>T was detected in the SACS gene. |
| BG   | Ataxia (upper and lower limb involvement), white matter abnormalities | Nd | Sporadic | CLCN2 (NM_004366.5) | c.218G>A; (p.Arg73His); het | Additional likely pathogenic heterozygous mutation c.1471C>T (p.Pro491Ser) was detected in the CAPN1 gene. |
| JO   | Ataxia, tandem gait, dystadiadochokinesia (mild), positional and action tremor, tremors, spine curvature, discopathy | Cortex and subcortical atrophy | Familial | CACNA1A (NM_001127221.1) | c.*652C>G; CLCN2 (NM_004366.5) | c.1730G>A; (p.Arg577Gln); het | The most suspected gene because of phenotypic concordance |
| OA   | Ataxia, gait and limb ataxia, action tremor, dysarthria, dysmetria, extensor plantar responses, pes cavus, nystagmus | Cerebellar vermis hypoplasia, cerebellar atrophy | Sporadic | SLC9A1 (NM_003047.4) | c.859G>A; (p.Gly287Ser); het | Autosomal recessive inheritance, no second mutation found |
| Case | Clinical features | Imaging | Family history | Gene (transcript) | Mutation | Comments |
|------|-------------------|---------|---------------|------------------|----------|----------|
| BT   | Cerebellar syndrome, gait ataxia, axonal motor neuron degeneration, dysmetria (mild), dysdiadochokinesia, swallowing problems, dysarthria, sphincter disturbances, wheelchair-bound, diabetes type 2 | Cortical cerebellar and brainstem atrophy, progressive supranuclear paralysis | Sporadic | REEP2 (NM_001271803.1) | c.605C>T; (p.Pro202Leu); het | Autosomal recessive or dominant inheritance, but gene related to spastic paraplegia type 72 |
|     | Age of onset 70 years | | | | | |
| GK   | Gait ataxia, ataxia, slurred speech, dysarthria, deep tendon reflexes, extensor plantar responses, cognitive impairment, hypotonia, dysmetria, dysdiadochokinesia, limb tremor, nystagmus (mild), thyroid insufficiency | Cerebellar atrophy | Sporadic | PIK3R5 (NM_001142633.2) | c.183G>C; (p.Gln61His); het | Autosomal recessive inheritance, no second mutation found |
|     | Age of onset 24 years | | | | | |
| MJ   | Toe walking, spastic gait, Achilles tendon | Normal MRI | Sporadic | SPTBN2 (NM_006946.2) | c.1741G>A; (p.Ala581Thr); het | Additional pathogenic heterozygous mutation c.2680C>T (p.Arg894Ter) was detected in the CLCN1 gene (included in other NGS panel) |
|     | Age of onset 7 years | | | | | |
| BW   | Gait ataxia, imbalance and incoordination, clumsiness, cerebellar dysarthria, scanning speech, rigidity, postural tremor, dysmetria and excessive reflexes of lower limbs, progressive lower limb weakness, hypertonia of limbs, axonal polyneuropathy, dysdiadochokinesia, bilateral extensor plantar responses | Cerebellar and brainstem atrophy, thinning of the corpus callosum | Familial | ITPR1 (NM_001168272.1) | c.2761G>A; (p.Gly921Ser); het | Negative NGS analyses because the genetic findings were inconsistent with a disease phenotype. Additional pathogenic heterozygous mutation c.305C>T (p.Pro102Leu) was detected in the PRKN gene (included in other NGS panel) |
|     | Age of onset 27 years | | | | | |
| DB   | Ataxia, gait ataxia, speech ataxia, bradykinesia (severe), problems with writing | Cortical and subcortical atrophy of the cerebellum and cerebellar vermis (severe) | Sporadic | PRKN (NM_012414.3) | c.1406C>T; (p.Ala469Val); het | Additional pathogenic heterozygous mutation c.823C>T (p.Arg275Trp) was detected in the PRKN gene (included in other NGS panel) |
|     | Age of onset 19 year | | | | | |
Application of the designed panel in ataxia patients revealed that the overall mutation detection rate amounted to 55.2% and varied from 33.3% in those with a familial history and autosomal dominant mode of inheritance (3/9 cases) to 53.3% among sporadic cases (8/15 cases). Among the latter cases, we identified variants in genes associated with disorders that are inherited in both autosomal recessive and autosomal dominant traits. The obtained results present significant molecular effectiveness in comparison with other NGS-based studies. For example, Németh et al. reported definite molecular diagnosis by gene panel sequencing in 18.0% of 50 probands with ataxia, although the detection rate varied from 8.3 to 40.0% depending on the age of symptoms onset (adult-, childhood-, or adolescent-onset disease) (Németh et al. 2013). Other studies using NGS exome sequencing revealed a diagnostic yield of 21.0% in a cohort of 76 patients with chronic progressive cerebellar ataxias and potential additional diagnoses in 40.0% (Fogel et al. 2014). A study of 319 cerebellar ataxia cases showed a yield of 22.6%, with possible additional diagnoses in 5.9% (Coutelier et al. 2018).

Due to comprehensive diagnostic investigations that involve clinical assessment and differentiate ataxia from acquired, primary cause, or secondary cause by examining the clinical findings, such as blood tests, neuroimaging tests, and genetic tests, a definitive molecular diagnosis of patients with suspected clinically hereditary ataxia often takes years and is highly complex.

In our study, the most frequently pathogenic mutations were located in the \textit{POLG}, \textit{CACNA1A}, \textit{SACS}, and \textit{SLC33A1} genes. Other variants were detected in the following genes: \textit{STUB1}, \textit{SPTBN2}, \textit{TGM6}, \textit{SETX}, \textit{ANO10}, and \textit{SPAST}. The most common type of mutation was missense mutation.

In the \textit{POLG} gene, we identified the two known pathogenic variants most frequently present in Caucasians: c.1399G>A (p.Ala467Thr) and c.2243G>C (p.Trp748Ser). These variants usually occur in the homozygous state and have high variability in their clinical presentation (Neeve et al. 2012; Van Goethem et al. 2004), which may be caused by genetic, epigenetic, and environmental factors (Neeve et al. 2012). Notably, our tested individual with a heterozygous mutation of c.1399G>A in the \textit{POLG} gene clinically presented with gait and limb ataxia, motor and sensory neuropathy, positive Romberg sign, decreased sensory nerve conduction velocity, impaired vibration sense in the right foot, cardiomyopathy, and age of onset of 70 years (patient RM). However, it was previously assumed that heterozygous carriers were unaffected cases (Neeve et al. 2012; Van Goethem et al.)

### Table 3 (continued)

| Case | Clinical features | Imaging | Family history | Gene (transcript) | Mutation | Comments |
|------|------------------|---------|----------------|------------------|----------|----------|
| NL   | Ataxia (more affected lower limbs), unstable gait, deep tendon reflexes, slurred speech, dysarthria, dizziness, lateral nystagmus, pes cavus | Cortical and subcortical atrophy of the cerebellum and cerebellar vermis (severe) | Familial | Negative NGS analyses because the genetic findings were inconsistent with a disease phenotype | POLG, CACNA1A, SACS, SLC33A1 | c.1399G>A (p.Ala467Thr) and c.2243G>C (p.Trp748Ser) | het, hom, homozgyous; Nd, not determined |
| Abbreviations: MRI, magnetic resonance imaging; het, heterozygous; hom, homozygous; Nd, not determined | | | | | | |

**Discussion**

Application of the designed panel in ataxia patients revealed that the overall mutation detection rate amounted to 55.2% and varied from 33.3% in those with a familial history and autosomal dominant mode of inheritance (3/9 cases) to 53.3% among sporadic cases (8/15 cases). Among the latter cases, we identified variants in genes associated with disorders that are inherited in both autosomal recessive and autosomal dominant traits. The obtained results present significant molecular effectiveness in comparison with other NGS-based studies. For example, Németh et al. reported definite molecular diagnosis by gene panel sequencing in 18.0% of 50 probands with ataxia, although the detection rate varied from 8.3 to 40.0% depending on the age of symptoms onset (adult-, childhood-, or adolescent-onset disease) (Németh et al. 2013). Other studies using NGS exome sequencing revealed a diagnostic yield of 21.0% in a cohort of 76 patients with chronic progressive cerebellar ataxias and potential additional diagnoses in 40.0% (Fogel et al. 2014). A study of 319 cerebellar ataxia cases showed a yield of 22.6%, with possible additional diagnoses in 5.9% (Coutelier et al. 2018).

Due to comprehensive diagnostic investigations that involve clinical assessment and differentiate ataxia from acquired, primary cause, or secondary cause by examining the clinical findings, such as blood tests, neuroimaging tests, and genetic tests, a definitive molecular diagnosis of patients with suspected clinically hereditary ataxia often takes years and is highly complex.

In our study, the most frequently pathogenic mutations were located in the \textit{POLG}, \textit{CACNA1A}, \textit{SACS}, and \textit{SLC33A1} genes. Other variants were detected in the following genes: \textit{STUB1}, \textit{SPTBN2}, \textit{TGM6}, \textit{SETX}, \textit{ANO10}, and \textit{SPAST}. The most common type of mutation was missense mutation.

In the \textit{POLG} gene, we identified the two known pathogenic variants most frequently present in Caucasians: c.1399G>A (p.Ala467Thr) and c.2243G>C (p.Trp748Ser). These variants usually occur in the homozygous state and have high variability in their clinical presentation (Neeve et al. 2012; Van Goethem et al. 2004), which may be caused by genetic, epigenetic, and environmental factors (Neeve et al. 2012). Notably, our tested individual with a heterozygous mutation of c.1399G>A in the \textit{POLG} gene clinically presented with gait and limb ataxia, motor and sensory neuropathy, positive Romberg sign, decreased sensory nerve conduction velocity, impaired vibration sense in the right foot, cardiomyopathy, and age of onset of 70 years (patient RM). However, it was previously assumed that heterozygous carriers were unaffected cases (Neeve et al. 2012; Van Goethem et al.)
The patient KW had the same variant in the POLG gene and also had the c.1506delA (p.Lys502AsnfsTer28) mutation in the SPAST gene. However, in this case, the mutation in the SPAST gene should be considered the more likely cause of the patient’s disease.

The gold standard in clinical practice should be the use of a focused approach for both NGS and Sanger sequencing. As an example, the homozygous splice site pathogenic mutation c.1218+1G>C in ANO10 was confirmed by Sanger sequencing in the proband and both parents in a consanguineous family. In addition, the analysis of cosegregation of the variants with the disease in families should always be performed when possible.

Interestingly, genetic analysis of two unrelated patients with suspected hereditary ataxia identified the heterozygous mutation c.1559T>C (p.Ile520Thr) in the SLC33A1 gene, which is related to autosomal dominant spastic paraplegia type 42 (Lin et al. 2008) or recessive congenital cataracts, hearing loss, and neurodegeneration (Hupke et al. 2012). To the best of our knowledge, the heterozygous missense mutation c.339T>G (p.Ser113Arg) in the SLC33A1 gene was identified in one Chinese family with autosomal dominant pure HSP (Lin et al. 2008). In addition, no mutations in SLC33A1 were detected among 220 Caucasian patients with autosomal dominant hereditary spastic paraplegias and negative for mutations in the SPAST gene (Schlipf et al. 2010). Our results may suggest that mutations in the SLC33A1 gene can be associated with spinocerebellar ataxia, hereditary spastic paraplegia, or both. However, further segregation analysis of gene variants in SLC33A1 and functional assays or analysis of a large group of ataxia patients should be performed.

Additionally, we assume that further reanalysis of the most likely variants, e.g., c.2062C>G (p.Pro688Ala) in the AFG3L2 gene, which ranges from being of unknown significance to pathogenic, may increase the diagnostic yield. Therefore, eventually, it may be extremely important to check for updates of the databases, i.e., HGMD, OMIM, ClinVar, and LOVD, related to gene variants that were previously classified as VUS. At present, these diagnoses cannot be unequivocally settled due to the lack of samples from parents.

The analysis of cosegregation of the variant c.146A>G in the STUB1 gene for the affected mother and cousin of the proband showed the presence of a heterozygous mutation in both, confirming that this variant cosegregates with SCA48. These genetic findings enable the renaming of this variant from VUS to pathogenic. Moreover, 128 affected patients tested for NGS did not reveal the presence of this mutation, which could be strong evidence for the presumed pathogenic variant. These findings together account for the pathogenicity of this variant in the STUB1 gene. Therefore, we propose that the most suspect VUS should be reported in the scientific literature because, with the growing knowledge in online databases, some gene variants can be reclassified from VUS to pathogenic.

The gene-specific NGS approach is subject to some limitations. The first limitation is the overlapping of neurological phenotypes and the presence of pathogenic mutations in genes encompassing other neurodegenerative disorders. The second limitation is the identification of more than one VUS per single case. The third limitation is the increasing information about novel variants in databases and the renaming of VUS as pathogenic variants. Thus, we do not exclude the possibility of a genetic cause of the disorder in patients with no known pathogenic variants detected in the SCA-SPG genes to date. In one such case, we were able to detect a deleterious heterozygous missense mutation of c.305C>T (p.Pro102Leu) in the PRNP gene that was associated with autosomal dominant Gerstmann-Straussler disease. A 32-year-old man (patient BW) presented with gait ataxia, progressive lower limb weakness, imbalance and incoordination, clumsiness, cerebellar dysarthria, scanning speech, rigidity, postural tremor, dysmetria, bilateral extensor plantar responses, hypertonia, dysdiadochokinesia, excessive reflexes of lower limbs, and axonal polyneuropathy, with an age at onset of 27 years old. MRI neuroimaging showed cerebellar and brainstem atrophy and thinning of the corpus callosum. The mutation was found through the analysis of different NGS panels that included 118 genes related to neurodegenerative and dementia disorders, which included the PRNP gene. However, we believe that the high detection rate confirmed that our strategy of using a targeted NGS approach that focuses on genes associated with both hereditary ataxias and hereditary spastic paraplegias was appropriate. This establishment is associated with the clinical and genetic overlapping of these two diseases, which is termed the ataxia-spasticity spectrum (Synofzik and Schüle 2017; Elert-Dobkowska et al. 2019).

Furthermore, together with single nucleotide variants, rare cerebellar ataxias are also caused by different types of mutations undetectable by NGS. Copy number variants (CNVs) in GRID2 are the main cause of SCAR18 (Ceylan et al. 2020). In addition, Friedrich’s ataxia is caused by non-coding GAA repeat expansion or ultrarare point mutations in the FXN gene. Recently, Cortese et al. (2019) identified a biallelic intronic pentanucleotide AAGGG repeat expansion in the RFC1 gene that is associated with cerebellar ataxia, neuropathy, and vestibular areflexia syndrome (CANSVAS) (Cortese et al. 2019). This finding expands the known molecular genetic basis of autosomal recessive cerebellar ataxia. All these examples can explain the likelihood of a lack of a definite molecular diagnosis in some ataxic patients after the application of only selected genetic tests.

In conclusion, we demonstrated that an approach based on a targeted NGS panel can be a highly effective and
useful tool in the final molecular genetic diagnosis of ataxia patients. Furthermore, we highlight that a sequencing panel that targets ataxias together with hereditary spastic paraplegia genes increases diagnostic success. Due to the complexity of the clinical picture and overlapping phenotypes between distinct neurological disorders, NGS testing is more common and is the gold standard in neurodegenerative disorder diagnostics.

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Author contribution The results presented in the manuscript will become a part of the student’s dissertation or thesis (Wiktoria Radziwonik).

AS and EE-D designed the study. AS, EE-D, and WR carried out the NGS study and data interpretation. WR wrote the manuscript with support from AS and EE-D.

AK-M, KZ-J, IS, and JZ provided clinical assessment and data helpful for NGS interpretation.

All authors discussed the results and contributed to the final manuscript.

Data availability All data relevant to the study are included in the article or uploaded as supplementary information. For further information, contact the corresponding author (suleka@ipin.edu.pl).

Declarations

Ethics approval and consent to participate The results of the study were obtained within the routine diagnostic procedures performed in accordance with the Good Laboratory Practice (GLP) and SOPs that are obligatory in IPiN. NGS data were interpreted according to the ACMG 2015 recommendations. Written consent forms were obtained from all patients.

Conflict of interest The authors declare no competing interests.

References

Angelini C, Van Gils J, Bigourdian A, Jouk PS, Lacombe D, Menegon P, Moutton S, Riant F, Sole G, Tourner-Lasserre E, Trainouille A, Vincent M, Goizet C (2019) Major intra-familial phenotypic heterogeneity and incomplete penetrance due to a CACNA1A pathogenic variant. Eur J Med Genet 62(6):103530. https://doi.org/10.1016/j.ejmg.2018.08.011

Bird TD (2019) Hereditary ataxia overview. 1998 Oct 28. In: Adam MP, Ardingr HH, Pagon RA et al. (eds) GeneReviews® [Internet]. University of Washington, Seattle, 1993-2022. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1138/

Bouhal Y, Zouari M, Kefi M, Ben Hamida C, Hentati F, Amouri R (2008) Autosomal recessive ataxia caused by three distinct gene defects in a single consanguineous family. J Neurogenet 22(2):139–148. https://doi.org/10.1080/01677060802025233

Brusco A, Geller C, Cagnoli C, Saluto A, Castucci A, Michielotto C, Feton V, Mariotti C, Migone N, Di Donato S, Taroni F (2004) Molecular genetics of hereditary spinocerebellar ataxia: mutation analysis of spinocerebellar ataxia genes and CAG/CTG repeat expansion detection in 225 Italian families. Arch Neurol 61(5):727–733. https://doi.org/10.1001/archneur.61.5.727

Ceylan AC, AcsarArslan E, Erdem HB, Kavus H, Arslan M, Topaloğlu H (2020) Autosomal recessive spinocerebellar ataxia 18 caused by homozygous exon 14 duplication in GRID2 and review of the literature. Acta Neurol Belg. https://doi.org/10.1007/s13760-020-01328-z

Cortese, A., Simone, R., Sullivan, R., Vandrovocova, J., Tariq, H., Yau, W. Y., Humphrey, J., Jaunmuktane, Z., Sivakumar, P., Polke, J., Ilyas, M., Tribollet, E., Tomaselli, P. J., Devigili, G., Callegari, I., Versino, M., Salpietro, V., Ethymiou, S., Kaski, D., Wood, N. W., … Houlden, H. (2019). Biallelic expansion of an intronic repeat in RFC1 is a common cause of late-onset ataxia. Nature genetics. 51(4), 649–658. https://doi.org/10.1038/s41588-019-0372-4

Coutelier, M., Hammer, M. B., Stevanin, G., Monin, M. L., Davoine, C. S., Mochel, F., Labague, P., Ewenyczky, C., Ding, J., Gibbs, J. R., Hannequin, D., Melki, J., Toutain, A., Laugel, V., Forlani, S., Charles, P., Brussolle, E., Thobois, S., Afenjar, A., Anheim, M., … Spastic Paraplegia and Ataxia Network (2018). Efficacy of exome-targeted capture sequencing to detect mutations in known cerebellar ataxia genes. JAMA neurology, 75(5), 591–599. https://doi.org/10.1001/jamaneurol.2017.5121

Denier C, Ducros A, Vahedi K, Joutel A, Thierry P, Ritz A, Castel-novo G, Deonna T, Gérard P, Devoize JL, Gayou A, Perrotty B, Soisson T, Autret A, Warter JM, Vighetto A, Van Bogaert P, Alamowitch S, Roulet E, Tourner-Lasserre E (1999) High prevalence of CACNA1A truncations and broader clinical spectrum in episodic ataxia type 2. Neurology 52(9):1816–1821. https://doi.org/10.1212/wnl.52.9.1816

Durr A (2010) Autosomal dominant cerebellar ataxias: polyglutamine expansions and beyond. The Lancet Neurol 9(9):885–894. https://doi.org/10.1016/S1474-4422(10)70183-6

Elert-Dobkowska E, Stepien I, Krysa W, Ziora-Jakutowicz K, Rakowicz M, Sobanska A, Pileh, J, Antczak-Marach D, Zaremba J, Sulek A (2019) Next-generation sequencing study reveals the broader variant spectrum of hereditary spastic paraplegia and related phenotypes. Neurogenetics 20(1):27–38. https://doi.org/10.1007/s40372-019-00565-6

Fogel BL, Lee H, Deignan JL, Strom SP, Kantarci S, Wang X, Quin-tero-Rivera F, Vilain E, Grody WW, Perlman S, Geschwind DH, Nelson SF (2014) Exome sequencing in the clinical diagnosis of sporadic or familial cerebellar ataxia. JAMA neurology 71(10):1237–1246. https://doi.org/10.1001/jamaneurol.2014.1944

Huppke P, Brendel C, Kalscheuer V, Korenc KGC, Marquardt I, Freisinger P, Christodoulou J, Hillebrand M, Pitelet G, Wilson C, Gruber-Seddmiyar U, Ullmann R, Haas S, Elpeelge O, Nürnberg G, Nürnberg P, Dad S, Moller L, Kaler SG, Gärnter J (2012) Mutations in SLC33A1 cause a lethal autosomal-recessive disorder with congenital cataracts, hearing loss, and low serum copper and ceruloplasmin. Am J Hum Genet 90(1):61–68. https://doi.org/10.1016/j.ajhg.2011.11.030

Lin P, Li J, Liu Q, Mao F, Li J, Qiu R, Hu H, Song Y, Yang Y, Gao G, Yan C, Yang W, Shao C, Gong Y (2008) A missense mutation in SLC33A1, which encodes the acetyl-CoA transporter, causes autosomal-dominant spastic paraplegia (SPG42). Am J Hum Genet 83(6):752–759. https://doi.org/10.1016/j.ajhg.2008.11.003

Neeve, V. C., Samuels, D. C., Bindoff, L. A., van den Bosch, B., Van Goethem, G., Smeets, H., Lombe, A., Jardel, C., Hirano, M., Dimao, S., De Vries, M., Smeintek, J., Smits, B. W., de Coo, I. F., Saft, C., Klopotock, T, Keiling, B. C., Czermin, B., Abicht,
A., Lochmüller, H., … Horvath, R. (2012). What is influencing the phenotype of the common homozygous polymerase-γ mutation p.Ala467Thr?. *Brain: a journal of neurology*, 135(Pt 12), 3614–3626. https://doi.org/10.1093/brain/aw298.

Németh, A. H., Kwasniewska, A. C., Lise, S., Parolin Schnekenberg, R., Becker, E. B., Bera, K. D., Shanks, M. E., Gregory, L., Buck, D., Zameel Cader, M., Talbot, K., de Silva, R., Fletcher, N., Hastings, R., Jayawant, S., Morrison, P. J., Worth, P., Taylor, M., Tolmie, J., O’Regan, M., … Ragoussis, J. (2013). Next generation sequencing for molecular diagnosis of neurological disorders using ataxias as a model. *Brain: a journal of neurology*, 136(Pt 10), 3106–3118. https://doi.org/10.1093/brain/awt236.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hege M, Lyon E, Spector E, Voelkerding K, Rehm HL, Laboratory Quality Assurance Committee ACMG (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine: Official Journal of the American College of Medical Genetics 17(5):405–424. https://doi.org/10.1038/gim.2015.30

Sarzi E, Bourdon A, Chrétienn D, Zahrane M, Corcos J, Slama A, Cormier-Daire V, de Lonlay P, Munnich A, Rötig A (2007) Mitochondrial DNA depletion is a prevalent cause of multiple respiratory chain deficiency in childhood. *J Pediatr* 150(5):531-534. e5346. https://doi.org/10.1016/j.jpeds.2007.01.044

Schlipf NA, Beetz C, Schüle R, Voelkerding K, Rehm HL, Laboratory Quality Assurance Committee ACMG (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine: Official Journal of the American College of Medical Genetics 17(5):405–424. https://doi.org/10.1038/gim.2015.30

Van Goethem G, Dermaut B, Löfgren A, Martin JJ, Van Broeckhoven C (2001) Mutation of POLG is associated with progressive exter- nal ophthalmoplegia characterized by mtDNA deletions. Nat Genet 28(3):211–212. https://doi.org/10.1038/90034

Van Goethem G, Luoma P, Rantamäki M, Al Memar A, Kaakkola S, Hackman P, Krahe R, Löfgren A, Martin JJ, De Jonghe P, Suolaitinen A, Udd B, Van Broeckhoven C (2004) POLG mutations in neurodegenerative disorders with ataxia but no muscle involvement. *Neurology* 63(7):1251–1257. https://doi.org/10.1212/01.wnl.0000140494.58732.83

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