Application of a custom NGS Gene Panel revealed a high diagnostic utility for molecular testing of hereditary ataxias.

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Abstract

Background

Hereditary ataxias (HA) are a rare group of heterogeneous disorders. Here, we present results of molecular testing a group of ataxia patients using custom-designed Next Generation Sequencing (NGS) panel. Due to genetic and clinical overlapping of hereditary ataxias and spastic paraplegias (HSP), designed panel encompassing together HA and HSP genes.

Methods

The NGS libraries comprising coding sequence for 152 genes were performed using KAPA HyperPlus and HyperCap Target Enrichment Kit and sequenced on the MiSeq instrument. Obtained results were analyzed using BaseSpace Variant Interpreter and Integrative Genomics Viewer. All pathogenic and likely pathogenic variants were confirmed using the Sanger sequencing.

Results

A total of 29 patients with hereditary ataxias were enrolled to the NGS testing, and 16 patients had a confirmed molecular diagnosis with diagnostic efficiency 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), *SPAST* (SPG4, n=1).

Conclusions

We demonstrated that approach based on targeted NGS panel can be highly effective and useful tool in the molecular diagnosis of ataxia patients. Furthermore, we highlight that sequencing panel targeted to ataxias together with HSP genes increase the diagnostic success.

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 genes with SCAR, 9 genes with EA, and 6 genes with SPAX. In addition, seven genes are known to be involved in X–linked cerebellar ataxias, and 4 genes 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 atrophy is a neuropathological and neuroimaging hallmark of ataxia.

Genetically, the molecular background of ataxias varies widely, ranging from point and small mutations to dynamic mutations. 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 possible expanding the phenotypic and genotypic spectrum of ataxias in clinical practice.

Materials And Methods

This study was approved by an Ethics Commission of IPiN. All individuals participating in the research signed the 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 in 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), electroneurography (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), ultrastructural imaging (1/29), ultrasound imaging of the abdomen (1/29) or skin and skeletal muscle biopsy (1/29).

The study was approved by the ethics commission of the Institute of Psychiatry and Neurology in Warsaw, Poland, and all participants gave written informed consent.

Methods

Genomic DNA was extracted from 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 was assessed by 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 postcapture 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 x 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 by using 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: (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, Leiden Open Variation Database (LOVD), and Human Gene Mutation Database (HGMD), whereas 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 – 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 diagnoses 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 – 28 years, 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 score of 68.1x. The mean proportion of sequence achieving 30X sequencing coverage was 95.5% and ranged from 89.6–97.2%.

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.

Table 2
Overview of definitive molecular diagnosis in 16 out of 29 Polish patients with clinical manifestations of hereditary ataxias.

Gene	Mutation	Molecular diagnosis	MOI	Prevalence [%]
POLG	(1) c.2615G>A (p.Ser872Asn) (2) c.1399G>A (p.Ala467Thr) observed in two patients	Progressive External Ophthalmoplegia with Mitochondrial DNA Deletions type 1	AD	3/16 [18.8%]
	(1) c.[428C>T](;)[2323G>A] p.(Ala143Val);(Glu775Lys) (2) c.[2243G>C](;)[2243G>C] p.(Trp748Ser);(Trp748Ser)	Spinocerebellar Ataxia with Epilepsy	AR	2/16 [12.5%]
CACNA1A	(1) c.2042_2043delAG (p.Gln681ArgfsTer103) (2) c.859T>C (p.Cys287Arg)	Episodic Ataxia type 2	AD	2/16 [12.5%]
SACS	(1) c.[5773_5779delCTGAGTG](;)[11374C>T] p.(Leu1925SerfsTer11);(Arg3792Ter) (2) c.[5498C>T](;)[7960T>G](;)[8340delT] p.(Ser1833Phe);(Tyr2654Asp);(His2781MetfsTer4)	Spastic Ataxia of Charlevoix-Saguenay	AR	2/16 [12.5%]
SLC33A1	c.1559T>C (p.Ile520Thr) observed in two patients	Spastic Paraplegia type 42	AD	2/16 [12.5%]
STUB1	c.146A>G (p.Tyr49Cys)	Spinocerebellar Ataxia type 48	AD	1/16 [6.3%]
SPTBN2	c.3824G>A (p.Arg1275Gln)	Spinocerebellar Ataxia type 5	AD	1/16 [6.3%]
TGM6	c.632G>A (p.Arg211His)	Spinocerebellar Ataxia type 35	AD	1/16 [6.3%]
SETX	c.[7252A>G](;)[7365_7366delTA] p.(Ser2418Gly);(Tyr2455Ter)	Ataxia-Oculomotor Apraxia Type 2	AR	1/16 [6.3%]
ANO10	c.[1218+1G>C](;)[1218+1G>C]	Spinocerebellar Ataxia type 10	AR	1/16 [6.3%]
SPAST	c.1506delA (p.Lys502AsnfsTer28)	Spastic Paraplegia type 4	AD	1/16 [6.3%]

Abbreviations: MOI, Mode Of Inheritance; AD, Autosomal Dominant; AR, Autosomal Recessive.

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 (Figure 1). The affected patient clinically presented with gait and limb ataxia, dysarthria, intention tremor and an 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 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 patients 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.

Table 3

Summary of VUS detected in 13 of 29 Polish patients by targeted NGS panel (without final molecular diagnosis).

Case	Clinical features	Imaging	Family history	Gene (Transcript)	Mutation	Comments
WS	4 limbs ataxia, gait ataxia, tandem gait, dysarthria, dystonia (torticollis, blepharospasm, laryngeal dystonia). Age of onset 22 years.	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. Age of onset 15 years.	Nd	familial	*CCDC88C* (NM_001080414.3)	c.5087T>C; (p.Leu1696Pro); het	Additional likely pathogenic heterozygous mutation c.660C>A (p.Tyr220Ter) were detected in *ACTA1* gene (included in different NGS panel).
PJ	Gait ataxia, involuntary movements, clumsiness, dyslexia, slurred speech, dysmetria (mild), action tremor, strabismus, axonal peripheral polyneuropathy, increased alpha-fetoprotein. Age of onset 10 years.	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, dysarthria, pyramidal signs. Age of onset 60 years.	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 were detected in *SACS* gene.
BG	Ataxia (upper and lower limb involvement), white matter abnormalities. Age of onset 56 years.	Nd	sporadic	*CLCN2* (NM_004366.5)	c.218G>A; (p.Arg73His); het	Additional likely pathogenic heterozygous mutation c.1471C>T (p.Pro491Ser) were detected in *CAPN1* gene.

Abbreviations: MRI, magnetic resonance imaging; het, heterozygous; hom, homozygous; Nd, not determined

Case	Clinical features	Imaging	Family history	Gene (Transcript)	Mutation	Comments
JO	Ataxia, tandem gait, dysdiadochokinesia (mild), positional and action tremor, tremors, spine curvature, discopathy. Age of onset 50 years.	Cortex and subcortical atrophy	familial	*CACNA1A* (NM_001127221.1)	c.*652C>G; het	The most suspected gene because of phenotypic concordance.
				CLCN2 (NM_004366.5)	c.1730G>A; (p.Arg577Gln); het	
OA	Ataxia, gait and limb ataxia, action tremor, dysarthria, dysmetria, extensor plantar responses, pes cavus, nystagmus. Age of onset 30 years.	Cerebellar vermis hypoplasia, cerebellar atrophy	sporadic	*SLC9A1* (NM_003047.4)	c.859G>A; (p.Gly287Ser); het	Autosomal recessive inheritance, no second mutation found.
BT	Cerebellar syndrome, gait ataxia, axonal motor neuron degeneration, dysmetria (mild), dysdiadochokinesia, swallowing problems, dysarthria, sphincter disturbances, wheelchair-bound, diabetes type 2. Age of onset 70 years.	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.
				B4GALNT1 (NM_001478.4)	c.728A>G; (p.Glu243Gly); het	Autosomal recessive inheritance, no second mutation found.
				SYNE1 (NM_182961.3)	c.14263C>T; (p.Leu4755Phe); het	
GK	Gait ataxia, ataxia, slurred speech, dysarthria, deep tendon reflexes, extensor plantar responses, cognitive impairment, hypotonia, dysmetria, dysdiadochokinesia, limb tremor, nystagmus (mild), thyroid insufficiency. Age of onset 24 years.	Cerebellar atrophy	sporadic	*PIK3R5* (NM_001142633.2)	c.183G>C; (p.Gln61His); het	Autosomal recessive inheritance, no second mutation found.
				KIF1A (NM_001244008.1)	c.2429C>T; (p.Thr810Met); het	Autosomal recessive or dominant inheritance, but gene related to spastic paraplegia type 30.
MJ	Toe walking, spastic gait, Achilles tendon. Age of onset 7 years.	Normal MRI	sporadic	*SPTBN2* (NM_006946.2)	c.1741G>A; (p.Ala581Thr); het	Additional pathogenic heterozygous mutation c.2680C>T (p.Arg894Ter) were detected in *CLCN1*

Abbreviations: MRI, magnetic resonance imaging; het, heterozygous; hom, homozygous; Nd, not determined.

Case	Clinical features	Imaging	Family history	Gene (Transcript)	Mutation	Comments
						gene (included in different NGS panel).
				USP8 (NM_005154.4)	c.3085G>A; (p.Asp1029Asn); het	
				C19orf12 (NM_001031726.3)	c.424A>G; (p.Lys142Glu); het	
				CYP7B1 (NM_004820.4)	c.929G>A; (p.Arg310Gln); het	
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. Age of onset 27 years.	Cerebellar and brainstem atrophy, thinning of the corpus callosum	familial	*ITPR1* (NM_001168272.1)	c.2761G>A; (p.Gly921Ser); het	Negative NGS analyses because of the genetic findings were inconsistent with a disease phenotype. Additional pathogenic heterozygous mutation c.305C>T (p.Pro102Leu) were detected in *PRNP* gene (included in different NGS panel).
				TDP1 (NM_018319.3)	c.1342C>T; (p.Arg448Trp); het	
				ATM (NM_000051.3)	c.4574T>C; (p.Ile1525Thr); het	
				RAB3GAP2 (NM_012414.3)	c.1406C>T; (p.Ala469Val); het	
DB	Ataxia, gait ataxia, speech ataxia, bradykinesia (severe), problems with writing. Age of onset 19 years.	Cortical and subcortical atrophy of cerebellum and cerebellar vermis (severe)	sporadic	Negative NGS analyses because of the genetic findings were inconsistent with a disease phenotype. Additional pathogenic heterozygous mutation c.823C>T (p.Arg275Trp) were detected in *PRKN* gene (included in different NGS panel).		
NL	Ataxia (more affected lower limbs), unstable gait, deep tendon reflexes, slurred speech, imbalanced, dysarthria, dysphagia, dizziness, lateral nystagmus, pes cavus, acanthocytosis. Age of onset 24 years.	Cortical and subcortical atrophy of cerebellum and cerebellar vermis (severe)	familial	Negative NGS analyses because of the genetic findings were inconsistent with a disease phenotype.		

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 for 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% in those with sporadic individuals (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 manners. The obtained results present significant molecular effectiveness in comparison to 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 8.3–40.0% 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 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 *POLG*, *CACNA1A*, *SACS* and *SLC33A1* genes. Other variants were detected in the following genes: *STUB1*, *SPTBN2*, *TGM6*, *SETX*, *ANO10* and *SPAST*. The most common type of mutation was missense mutation.

In the *POLG* gene, we identified 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 *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 an 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., 2004]. 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 using both a focused approach for 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 [Huppke 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*, 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 range from 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 co-segregation of the variant c.146A>G in the *STUB1* gene for the affected mother and cousin of the proband (Figure 2) showed the presence of a heterozygous mutation in both, confirming that this variant co-segregates 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 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, axonal polyneuropathy and 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 clinical and genetic overlapping of these two diseases, which is termed 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 a main cause of SCAR18 [Ceylan et al., 2020]. In addition, Friedreich's ataxia is caused by noncoding GAA repeat expansion or ultrarare point mutations in the *FXN* gene. Recently, Cortese A. 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 (CANVAS) [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 select 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.

Statements And Declarations

Funding:

No funding was received to conduct this study.

Conflict of Interest/Competing Interests:

The authors have no conflicts (financial or non-financial) of interest to declare that are relevant to the content of this article.

Ethics approval and Consent

The results of the study were obtained within the routine diagnostic procedures performed in accordance to 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.

Data availability statement

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).

Author Contribution Statement

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

Anna Sulek, and Ewelina Elert-Dobkowska designed the study; Anna Sulek, Ewelina Elert-Dobkowska and Wiktoria Radziwonik carried out the NGS study and data interpretation; Wiktoria Radziwonik wrote the manuscript with support from Anna Sulek and Ewelina Elert-Dobkowska,

Aleksandra Klimkowicz-Mrowiec, Karolina Ziora-Jakutowicz, Iwona Stepniak and Jacek Zaremba provided clinical assessment and data helpful for NGS interpretation;

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

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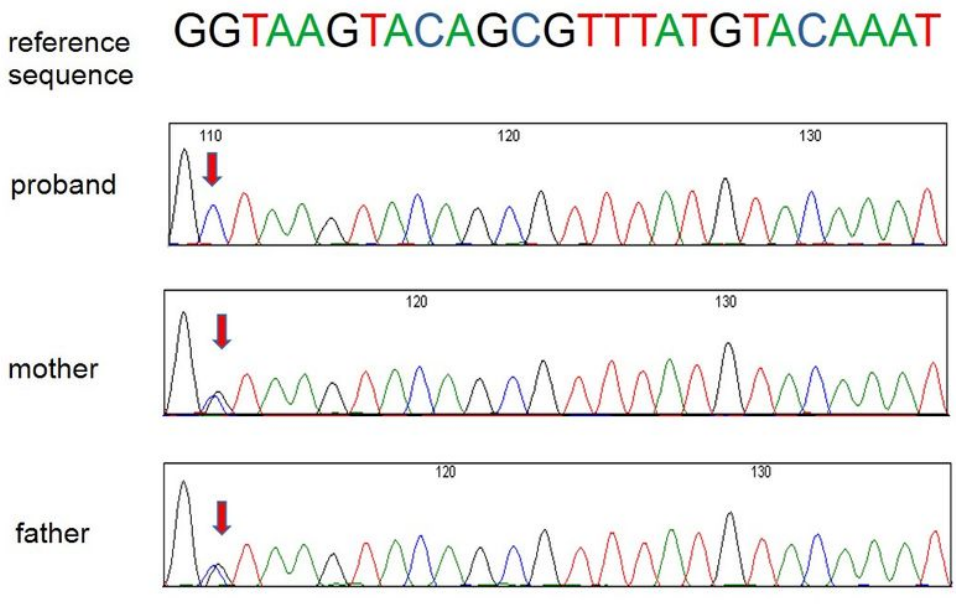
Tables

Due to technical limitations, Table 1 is only available as a download in the Supplemental Files section.

Figures



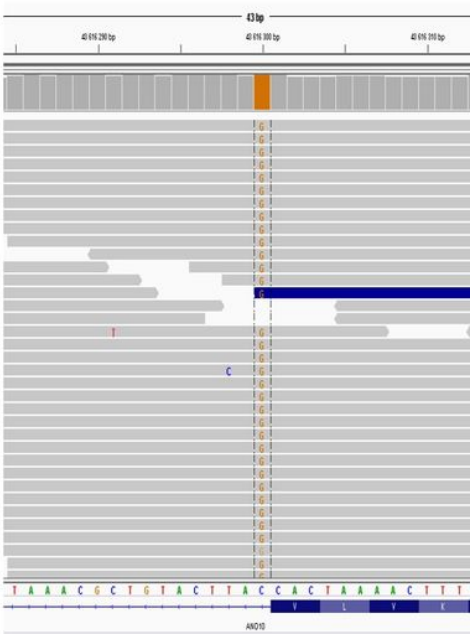


Figure 1

Analysis of cosegregation of the homozygous c.1218+1G>C mutation in ANO10 with autosomal recessive spinocerebellar ataxia type 10 in the family. To present and confirm the mutation, Integrative Genomics Viewer (IGV) software and Sanger sequencing were performed.

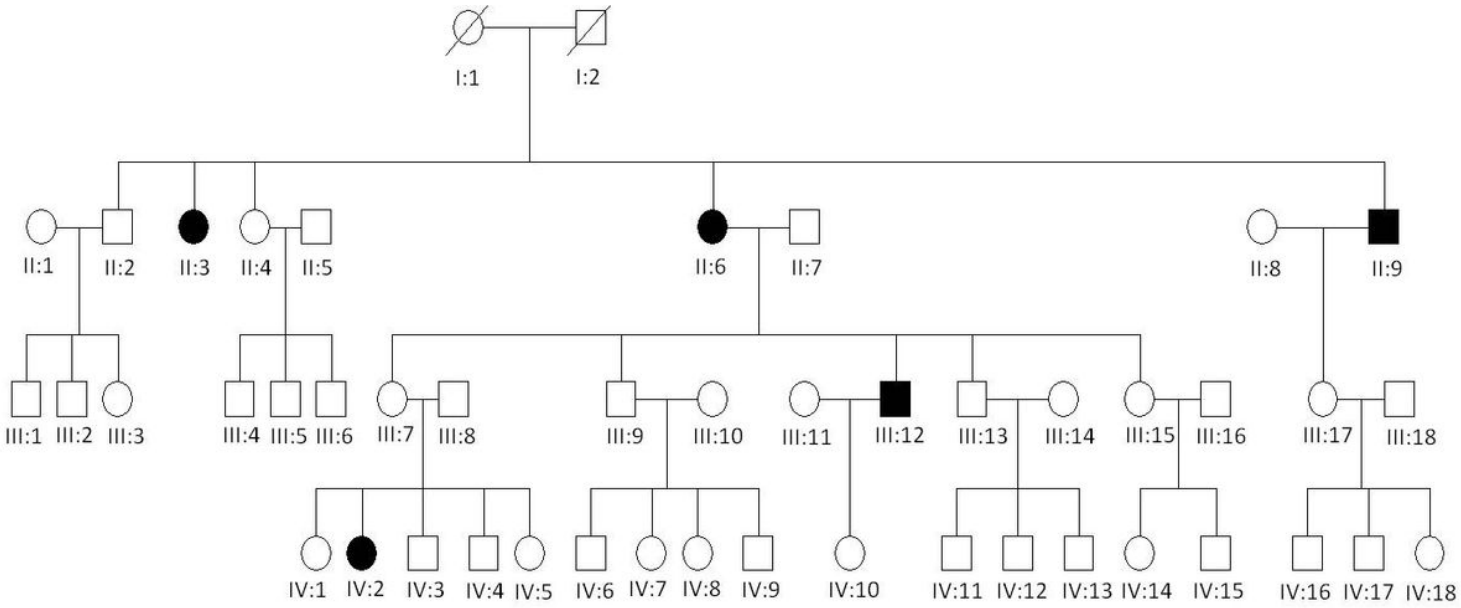


Figure 2

Pedigree of dominant phenotype determined by a heterozygous mutation of c.146A>G (p.Tyr49Cys) in the STUB1 gene. For three individuals, II:6, III:12 and IV:2, Sanger sequencing was carried out and revealed cosegregation of variants within the family. Abbreviations: squares indicate males; circles – females; an arrow – propositus; slash marks – deceased cases.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table1.doc
- SupplTable1.xlsx