Prevalence and phenotype of the c.1529C>T SPG7 variant in adult-onset cerebellar ataxia in Italy

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Abstract

Background: Hereditary Ataxias are heterogeneous groups of neurodegenerative disorders, characterized by cerebellar syndrome associated with dysarthria, oculomotor and corticospinal signs, neuropathy and cognitive impairment.

Recent reports suggested mutations in the *SPG7* gene, causing the most common form of autosomal recessive spastic paraplegia (MIM#607259), as a main cause of ataxias. The majority of described patients, were homozygotes or compound heterozygotes for the c.1529C>T (p.Ala510Val) change. We screened a cohort of 895 Italian ataxic patients for the p.Ala510Val in order to define the prevalence and genotype - phenotype correlation of this variant.

Methods: We set up a rapid assay for c.1529C>T using restriction enzyme analysis after PCR amplification. We confirmed the diagnosis with Sanger sequencing.

Results: We identified eight homozygous and 13 compound heterozygotes, including two novel variants affecting splicing.

Mutated patients showed a pure cerebellar ataxia at onset, evolving in mild spastic ataxia (alternatively) associated with dysarthria (~80% of patients), urinary urgency (~30%), and pyramidal signs (~70%). Comparing homozygotes and compound heterozygotes we noted a difference in age at onset and SARA scale between the two groups, supporting an earlier and more severe phenotype in compound heterozygotes vs. homozygotes.

Conclusion: *SPG7* c.1529C>T (p.Ala510Val) mutants account for 2.3% of cerebellar ataxia cases in Italy, suggesting that this variant should be considered as priority test in the presence of late onset pure ataxia. Moreover, the heterozygous/homozygous genotype appears to predict the onset of clinical manifestation and disease progression.

Introduction

Hereditary Ataxias (HA) and Hereditary Spastic Paraplegias (HSP) are clinically, genetically, and etiologically heterogeneous neurodegenerative disorders. HA are characterized by a cerebellar syndrome causing progressive loss of coordination with gait and limb ataxia, accompanied by dysarthria and oculomotor signs [1]. Conversely, HSP main features include the dysfunction of the corticospinal tract and dorsal columns, which results in spasticity and weakness in the lower limbs, in the absence of any additional neurological sign [2]. Spasticity and cerebellar ataxia are a relatively common combination, as in HSP due to *SPG7* mutations or occur in disorders that have specifically been designated spastic ataxia (SPAX) [3]. In the past years, several HSP families have been described, showing cerebellar features such as ataxia, suggesting a genetic overlap between those two neurodegenerative disorders [4, 5].

To date, more than 200 genes have been reported, encompassing recessive, dominant and X-linked HA/HSP forms [1](http://neuromuscular.wustl.edu/ataxia/aindex.html). Despite this huge number of ataxias and paraplegias described, more than half of all patients remain genetically undiagnosed. Among the most interesting examples of genes involved in both HSP and HA are *SPG7* and *AFG3L2*, encoding for the components of the mitochondrial AAA protease (*m*-AAA). Paraplegin and AFG3L2 have been implicated in a very broad range of neurodegenerative diseases, including pure ataxia [6] myoclonus or ataxia plus progressive myoclonic epilepsy [7, 8], ataxia and progressive external ophthalmoplegia (PEO) [9], ataxia plus type-1 muscle fiber atrophy [10], and optic atrophy [11-14].

A recent report suggested that mutations in *SPG7*, causing the most common form of autosomal recessive spastic paraplegia (MIM#607259), account for approximately 19% of cases in a group of 70 patients with unexplained ataxia and subtle pyramidal signs in UK [13]. All patients were homozygotes or compound heterozygotes for the c.1529C>T (p.Ala510Val) missense mutation [13]. The role of c.1529C>T as pathogenic variant has been doubtful for many years, given its high frequency in the healthy population. This variant localizes to the C-terminal end of

the protein, on the buried surface of α6 facing the α5, and a valine in this position would disturb the hydrophobic core interactions in the helical bundle, due to its wider volume [15]. Moreover, yeast complementation assays, have provided evidence that p.Ala510Val variant is not able to restore the respiratory competence of *yta10Δyta12Δ* cells and the proteolytic cleavage of the substrate protein Mrpl32, suggesting a pathogenic effect of this change [16].

Here, we evaluated the role of p.Ala510Val change in ataxia, screening a large cohort of 895 undiagnosed unrelated ataxic patients of Italian ancestry.

Materials and methods

Subjects

We enrolled 895 unrelated cases referred to the Genetics Unit in "Città della Salute e della Scienza" Hospital, Torino, Dept. of Neuroscience, Psychology, Drug Research and Child's Health, University of Florence, or the Neurogenetic Unit, IRCCS Stella Maris, Pisa. Four hundred thirty-five were males and 460 females, with a median age at first examination of 55.3 years (range 13-84 years). Clinically, all patients presented with ataxia or gait disturbance at onset, variably associated with dysarthria, dysphagia or oculomotor signs. Scale for the assessment and rating of ataxia (SARA score) was used to evaluate clinical features. In all, secondary causes of ataxia had previously been excluded by appropriate routine. Based on clinical features and appropriate gene testing, we ruled out a diagnosis of SCA1-3/6/8/12 and Friedreich ataxia in all the patients. Forty-four patients were referred with a familiar history, 24 suggesting a dominant inheritance and 20 a recessive inheritance. The remaining were sporadic cases.

Two hundred and fifty-seven adult healthy volunteers of Italian ancestry (122 males, 135 females; mean age±SD = 67.8±7.9 years) were screened in order to verify the frequency of the c.1529C>T-(p.Ala510Val) allele in control population. Informed consent was obtained from all participants and the internal Ethics Committee of the Department of Medical Sciences, University of Torino, Italy approved the study.

Mutation detection

We set up a rapid restriction enzyme assay for typing the c.1529C>T (p.Ala510Val) variant. Sanger sequencing was used to confirm the presence of the variant and to search for the second variant in compound heterozygotes. Details are available in supporting information. MLPA analysis for *SPG7* was performed using the SALSA MLPA P213 HSP in the conditions specified (www.mlpa.com).

Results

SPG7 p.Ala510Val mutants account for 2.3% of cerebellar ataxia cases in Italy.

Among the 895 patients screened using the restriction enzyme test, we identified eight homozygous and twenty-four heterozygotes cases harboring the c.1529C>T change. To search for a pathogenic mutation on the second allele, we sequenced the entire *SPG7* coding region in all heterozygotes. In thirteen, we found a second variant (TableS2, supporting information): seven of these were already reported as pathogenic changes (c.1A>G/p.Met1?; c.637C>T/p.Arg213*; c.773_774delTG/p.Val258Glyfs*30; c.1369C>T/p.Arg457*; c.1617delC/p.Val540Cysfs*52; c.2102A>C/p.His701Pro; c.2228T>C/p.Ile743Thr) (see references in supporting information). Five genetic changes were novel: in ATA-23, we identified the c.1877T>C substitution, that changed the conserved methionine 626 into a threonine; ATA-16 and ATA-17 showed the c.1972G>A variant, causing p.Ala658Thr. These variants are predicted as pathogenic: both affect amino acids in a highly evolutionary conserved region of the protein (from humans to *C.elegans*), are absent in the gnomAD and 1000G databases, and classified as pathogenic (IIIb) following ACMG guidelines (Table 1).

In ATA-25, the c.2181G>A mutation hits the last base of exon 16, causing a synonymous amino acid change. Splicing prediction tools, however, support an effect on canonical donor splice site. The remaining two novel variants are intronic: c.287-1G>C in ATA-14 and c.1553-2_1553-1delAG in ATA-15. *In-silico* prediction software showed that the c.287-1G>C substitution and the deletion

c.1553-2_1553-1delAG caused the loss of the canonical acceptor site. Unfortunately, cDNA was not available to confirm splicing effects.

In the remaining 11 carriers without biallelic mutations, we excluded *SPG7* single and multiple exon deletion/duplication by MLPA analysis. We also tested variants in exons 10-14-15 and 16 of the *AFG3L2* (where the majority of mutations are clustered), because of the role of its protein as partner of paraplegin in *m*-AAA complexes, without revealing any positive case.

Overall, p.Ala510Val homozygotes were 10/25 (40%, 8 families), and compound heterozygotes 15/25 (60%, 13 families).

In the healthy volunteers' cohort, we observed only two out of 257 individuals carrying the heterozygous c.1529C>T variant, consisting in an allelic frequency of 0.38%. No second variant was detected in the rest of the gene by direct Sanger sequencing and MLPA analysis.

Clinical features of SPG7-related ataxia cases

Clinical features of the twenty-five SPG7 patients (21 families) are summarized in Table 2 and detailed in supporting information. Ataxia or gait disturbances were often reported as initial symptoms. In family R, the disease started with pure dysarthria, with ataxia and dysarthria in families F, Q and T, and with limb spasticity in families J, S and V.

The median age at onset was 44.5±8.54 years (mean±SD). Comparing homozygotes and compound heterozygotes, we noted a difference between age at onset in the two groups: 50.6±7.40 vs. 40.7±7.00 (mean±SD; p=0.0023, Student's t-test; table 2). Moreover, SARA scale at onset revealed a difference between the two groups: 6.2±2.2 vs. 9.7±3.6 (mean±SD, p=0.03, Mann-Whitney test). This difference is maintained comparing age at onset of our homozygous individuals with classical SPG7 patients from literature, negative for p.Ala510Val allele (age at onset 50.6±7.40 vs. 34.8±12.8, p=0.0004) [12-14, 17-22].

Brain MRI at onset presented a cerebellar hemispheric and/or vermian atrophy in 18 out of 25 patients. Cerebellar dysarthria was also manifested in 68% of patients and 32% of patients showed

oculomotor signs (5/26 ophthalmoparesis, 1/26 slow saccades and 2/26 nystagmus). Pyramidal signs were present in ~76% of patients, in particular hyperreflexia (19/25), and spasticity (16/25). About 40% of patients showed a defect in proprioceptive sensation. Only three patients presented with urinary urgency at onset.

On follow up examination (mean 12.5 years of disease duration), 20/23 patients showed hyperreflexia and 18/23 spasticity. MRI studies identified a marked hemispheric and vermian atrophy of cerebellum in ATA-12 and ATA-25. Nystagmus initially reported in patients ATA-3 and ATA-13 progressed in ophthalmoparesis. Ten out of 23 patients showed urinary urgency. SARA scores became more similar between the two groups; however, values rating stance at follow up maintained a difference (mean±SD, 1.44±1.0 vs. 2.67±1.5; p=0.012, Mann-Whitney test).

Discussion

The huge genetic heterogeneity of HA and HSP is accompanied by allelic heterogeneity, given that the same gene can be associated with HA or HSP or mixed phenotypes. One such example, is given by *SPG7*: indeed, while *SPG7* complete loss of function seems associated with HSP type 7, likely hypomorphic variants, such as the p.Ala510Val, have been repeatedly described in HA.

To further study the role of p.Ala510Val, we collected and screened 895 Italian cases from different regions using an in house developed rapid genetic test. This survey represents the largest group of ataxic patients so far analyzed for this variant.

We identified 25 cases from 21 families (2.3%), homozygous or compound heterozygous for p.Ala510Val and another pathogenic variant in *SPG7*.

These patients presented with an ataxic phenotype at onset, explaining why *SPG7* was not considered for the initial screening. As expected, the majority of cases are negative for familiar history: only four autosomal recessive cases (A, F, Q and R) were reported.

Overall, *SPG7*-related cerebellar ataxias in our cohort has lower frequency when compared to previously reported survey from other populations, ranging from 18.6% in British patients to

19.3% in French-Canadian cases [13, 19]. This frequency may indicate a lower prevalence of the variant in SPG7 patients of Italian ancestry. Indeed, in our control cohort of 257 healthy individuals of Italian origin we only identified two p.Ala510Val alleles (0.4%), corroborating the results obtained by Arnoldi et al. in their analysis of HSP cases in Italian families [23].

Although a precise estimate of age at onset is difficult in HA and HSP, we confirm the variable age of onset (mean 44.5±8.54 years, range 29-63) which was always in adulthood. We noted a difference comparing the age at onset of homozygotes and compound heterozygotes individuals, with the former having the disease approximately ten years later than the latter (p=0.002). This difference is maintained comparing age at onset of our homozygous individuals with classical SPG7 patients from literature, negative for p.Ala510Val allele (age at onset 50.6±7.40 vs. 34.8±12.8, p=0.0004) [12-14, 17-22]. Even if interesting, these differences need further confirmation in larger surveys.

Using SARA rating scale, we showed p.Ala510Val homozygotes have a milder phenotype at onset vs. compound heterozygotes (p=0.03). At follow up (disease duration 13.7yrs in homozygotes vs. 11.7yrs in compound heterozygotes), homozygotes and compound heterozygotes are indistinguishable except for stance. Indeed, the majority of compound heterozygotes patients are no more able to stand with feet together without sway, whereas homozygotes have lost only the ability to stand in tandem. These results are in agreement with the less severe effect of p.Ala510Val compared to others variants, and may help explaining why this allele is so frequent in several populations. Thus, SPG7 complete loss-of-function seems associated with a more severe phenotype while a partially residual function seems associated with a milder phenotype.

Given its mild effect, it is possible that other genes play a role in cases with p.Ala510Val. Among these, *AFG3L2* is the most likely candidate, being its protein a component of the *m*-AAA heterohexamer with paraplegin. Thus, we may speculate that in cases with p.Ala510Val, the phenotype is modulated by variants acting on SPG7/AFG3L2 expression, or on AFG3L2 itself. A role for *AFG3L2* as modifier has already been proved by our group in a family with AOA2 [7].

In addition to p.Ala510Val, we also found thirteen other variants in compound heterozygous state. In ATA-13, we identified the c.2102A>C, causing the change of a highly conserved histidine in position 701 to a proline, within the catalytic metallopeptidase domain of paraplegin. This mutation was previously described only in Norwegian families, residing on a founder haplotype [24].
In ATA-24, we found the truncating mutation c.637C>T, p.Arg213*, already reported in a sporadic case of Amyotrophic Lateral Sclerosis (ALS). As suggested by the authors, this is not surprising, given that ALS and SPG7 share common clinical features, and might therefore represent a differential diagnosis [25].
We also reported five novel SPG7 mutations: two missense (p.Met626Thr, p.Ala658Thr), a single synonymous amino acid change (p.Ala727Ala), that likely affects canonical donor splice site, and two intronic variants affecting canonical acceptor splice site (c.287-1 G>C, c.1553-2_1553-1delAG) falling in the IIIb group, as pathogenic.

In eleven patients, the second variant could not be detected likely because of our *SPG7* testing had limitations. We excluded point mutations in coding regions and exon boundaries (±50bp), and intragenic deletion/duplication. Regulatory variants or deep intronic changes affecting splicing were not searched for in our study, therefore the analysis of these regions could be a promising approach in the identification of unreported but pathogenic variants.
Moreover, driven by their participation in *m*-AAA complexes, we hypothesized a role for *AFG3L2* as phenotype modifier. Unfortunately, no variants were found, confirming data obtained by [12].
In conclusion, we confirmed that cerebellar ataxia can be the main clinical symptom at onset in HSP type 7, and, therefore, *SPG7* should be considered in the differential diagnosis of sporadic and recessive late-onset ataxias, also in the Italian population. Given the frequency of the c.1529C>T allele, where access to reasonably cheap panels is not available, one could consider doing a tiered approach in sporadic/recessive ataxias and test this variant after the exclusion of FRDA and the most common SCAs.

Moreover, genotype appears to predict onset of clinical manifestations and disease progressions further elements worth of consideration when counseling the patients.

Disclosure of Conflicts of Interest: None.

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Table 1. Mutation pathogenicity prediction by *in-silico* tools.

Mutation	Evolutionary Conservation	ExAC	Mutation Taster	Effect on Protein Sequence	Domains lost	References
c. 637C>T, p.Arg213*	Medium	14/ 121412	Disease Causing	73% of sequence lost NMD	AAA Peptidase	Kruger 2016
c.773_774del, p.Val258Glyfs*30	Very high	-	Disease Causing	67% of sequence lost NMD	AAA Peptidase	Casari 1998
c.1369C>T, p.Arg457*	Very high	2/ 121312	Disease Causing	42% of sequence lost NMD	AAA (part) Peptidase	Klebe 2012
c.1617delC, p.Val540Cysfs*52	High	2/ 119946	Disease Causing	32% of sequence lost NMD	Peptidase	Arnoldi 2008
c.2181G>A, p.?	Medium	1/ 107032	Disease Causing	8% of sequence lost -	Peptidase (part)	-

Mutation	Evolutionary Conservation	SIFT	Polyphen-2	I-Mutant 2.0	Mutation Taster	PMUT	PON-P	PhD-SNP	ExAC	References
c.1529C>T, p.Ala510Val	Very High	Affects Protein Function (0.00)	Probably Damaging (1.000)	Increased Stability	Disease Causing	Disease (0.86)	Unknown (0.785)	Disease	306/ 121348	Pfeffer 2015 Roxburgh 2013 Sanchez-Ferrero 2013
c.1A>G, p.Met1?	?	Affects Protein Function (0.00)	Probably Damaging (0.713)	Decreased Stability	Disease Causing	Neutral (0.23)	Neutral (0.165)	Neutral	-	Klebe 2012
c.2102A>C, p.His701Pro	Medium	Tolerated (0.21)	Benign (0.260)	Increased Stability	Disease Causing	Disease (0.73)	Pathogenic (0.833)	Disease	4/ 117698	Wedding 2014 Rydning 2016
c.2228T>C, p.Ile743Thr	High	Affects Protein Function (0.01)	Probably Damaging (0.976)	Decreased Stability	Disease Causing	Disease (0.86)	Pathogenic (0.802)	Disease	6/ 120874	Brugman 2008
c.1877T>C, p.Met626Thr	Very High	Tolerated (0.26)	Probably Damaging (1.000)	Decreased Stability	Disease Causing	Neutral (0.49)	Pathogenic (0.982)	Neutral	-	-
c.1972G>A, Ala658Thr	Very High	Tolerated (0.15)	Probably Damaging (1.000)	Decreased Stability	Disease Causing	Disease (0.56)	Pathogenic (0.934)	Disease	-	-

Note: Bioinformatics tools used to analyze the impact of changes on paraplegin protein structure/function are reported in supplemental information.

Table 2 – Main clinical features in *SPG7* patients at first examination and follow up.

Family	Code	Gender	A510V Genotype	Age at first exam	Symptom at onset	SARA score	Spasticity	Follow up (yrs)	SARA score at follow up	Spasticity at follow up
A	ATA-1	**M**	Homoz	60	G	7	+	10	13.5	+
A	ATA-2	**M**	Homoz	56	G	2	+	10	7	+
B	ATA-3	**M**	Homoz	54	G	6	-	10	10	+
C	ATA-4	**M**	Homoz	60	G		-			
D	ATA-5	**M**	Homoz	65	G	8	+/-	5	10.5	+/-
E	ATA-6	**M**	Homoz	60	G	6.5	-	11	10	-
F	ATA-7	**M**	Homoz	38	G+D	6	-	2	7	+/-
F	ATA-8	**F**	Homoz	35	G+D	8	+/-	2	11	
G	ATA-9	**M**	Homoz	51	G	4	+ LL	5	6.5	+
H	ATA-10	**M**	Homoz	74	G	9	+	0.5	9	+
I	ATA-11	**F**	Compound Heteroz	66	G	12	+	8	14	+
J	ATA-12	**M**	Compound Heteroz	55	G+R	5	+/-	7	8	++
K	ATA-13	**M**	Compound Heteroz	48	G	14	-	13	31	+
L	ATA-14	**M**	Compound Heteroz	51	G	5	-	10	6	-
M	ATA-15	**M**	Compound Heteroz	49	G	14	-	1	NA	-
N	ATA-16	**F**	Compound Heteroz	58	G	4	+ LL	3	4	+
O	ATA-17	**F**	Compound Heteroz	48	G	12	+	1	12	+
P	ATA-18	**F**	Compound Heteroz	55	G+D	14	+			
P	ATA-19	**F**	Compound Heteroz	53	G+D	13	+			
Q	ATA-20	**F**	Compound Heteroz	40	D	9	-	2	10	+/-
Q	ATA-21	**M**	Compound Heteroz	41	D	8	-	0.5	8	+
R	ATA-22	**F**	Compound Heteroz	43	R	10	++	1	11	++
S	ATA-23	**F**	Compound Heteroz	45	G+D	9	+	1	10	+
T	ATA-24	**M**	Compound Heteroz	50	G+R	11	++	3	13	++
U	ATA-25	**F**	Compound Heteroz	46	G	5	+	6	8	+

Abbreviations: G: impairment of gait; D: Dysarthria; R: Rigidity; UL: Upper Limbs; LL: Lower Limbs; NA Not Available

Supporting Information

Materials and Methods

c.1529C>T/p.Ala510Val variant detection by restriction enzyme assay

Genomic DNA was extracted from whole-blood samples derived from patients and healthy volunteers, using standard methods.

To create an *Rsa*I site of cleavage in the presence of c.1529T allele, we designed an antisense mismatched primer (Table S1-A). PCR was performed under the standard conditions of the KAPA2G fast HotStart DNA polymerase (KAPA Biosystems, Roche, Mannheim, Germany). The *Rsa*I endonuclease cleavage was performed at 37°C for 3 hrs (New England Biolabs, Ipswich, MA, USA), followed by 3% agarose-TBE 1x gel electrophoresis. Expected bands for wild-type and mutated alleles were 162 bp and 140 bp, respectively. Sanger sequencing confirmed PCR-RFLP results.

SPG7 sequence analysis

We amplified the 17 coding exons of the *SPG7* gene (NM_003119) using primers and conditions reported in table S1-B. Amplicons were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientifics, Carlsbad, CA, USA) following manufacturer's instructions. The sequence products were run on an ABI 3130XL and analyzed using the SeqSCape 2.5 software (Applied Biosystems).

Multiplex ligation-dependent probe amplification (MLPA) analysis was carried out using MLPA P213-HSP mix-2 kit, according manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). Electrophoresis was performed using ABI 3130xl and ABI 3730 Sequencers and data analyzed with GeneMapper 3.5 software (Applied Biosystems) and Coffalyser.Net software (MRC-Holland).

AFG3L2 analysis

Exon 10-14-15 and 16 of *AFG3L2* (encompassing SCA28 mutations so far described) were amplified and sequenced using oligonucleotides and conditions reported in table S1-C.

Statistical and bioinformatics analyses

Statistical analysis was performed with the two-tails Student's t-test or Mann Whitney Test, using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Identifications of mutations and consequences on the protein in the case of the novel variants were interpreted using the following softwares and databases: Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/), gnomAD (gnomad.broadinstitute.org), Mutation T@ster (http://www.mutationtaster.org/), the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html), ESE finder 3.0 (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), SIFT (http://sift.bii.a-star.edu.sg/) and PoliPhen-2 (http://genetics.bwh.harvard.edu/pph2/). Genetic Variant Interpretation Tool was used to evaluate variant classification [26].

References of materials and methods and table 1 [12, 23-25, 27, 28]**.**

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Supp. Table S1: Oligonucleotide used.

A. Primers for mutagenesis and PCR conditions

A510V_F	5'- CGCACCTGTGGCAGTAACTA	60
A510V_R	5'- ACTGAATCCTGGTGTCAGCTGT	

B. Primers, PCR conditions for *SPG7* mutation screening

Exon (size)	**Primers (Forward/Reverse)**	**PCR Ta (°C)**
1 (337bp)	1F: 5'- CGCAGGCGCCACGTCAGA 1R:5'- GCCGGGCTGGGCCTTACAGA	56
2 (419bp)	2F: 5'- TGTTACCTAAAGCTTTGACCTATTG 2R:5'- GCTCTGATCACACCATTGTACTGC	56
3 (252bp)	3F: 5'- ACACTGTTGTCCTGTATGCCTCC 3R:5'- TCCAGACTGGTTTCACCTTGCTA	56
4 (378bp)	4F: 5'- GATGTCGCCCGTGTCTGTTG 4R:5'- CCACAGCCTCACTCTCACAGG	56
5 (280bp)	5F: 5'- GGCTCTCTGTTGACTGTAGGGTTG 5R:5'- TCTGTTTCTCAGATTACAAAGCCAA	56
6 (336bp)	6F: 5'- CGTAGGGATTCCTCGTCTCATCT 6R:5'- TTCAGGCTACTCTCTGCAACAGG	56
7 (256bp)	7F: 5'- GCATCGTGCTGCTGATTTCC 7R:5'- GAGCCCTTCTGGGAGAGGAG	56
8 (323bp)	8F: 5'- CGTGACCCAGAGAGACCTTACCT 8R:5'- ACACCAGAGGAAGGATGTGTGAA	56
9 (313bp)	9F: 5'- GGGTACAGGAAGAGGCTTTGTTT 9R:5'- CAACCTGTTCTGAAAGACATCGG	56
10 (321bp)	10F: 5'- CTCTCTCCCTCCTGTGTCCTG 10R:5'- GGCTTCACACCAAGAAGTGTCTTA	56
11 (243bp)	11F: 5'- CGCACCTGTGGCAGTAACTA 11R:5'- AGGCCTCGATGCTGTTTG	56
12 (250bp)	12F: 5'- TCCTCCTCTTAAGCCCTGATAGC 12R:5'- TCAATACCTGCCTGGGTATTTCT	56
13 (377bp)	13F: 5'- CTGGTCTCGAACTCCTGTCCTCAG 13R:5'- AGGCTTTCCTCTCACATGACCTACA	56
14 (244bp)	14F: 5'- GCATCCTGCCTACTGACCTG 14R:5'- GAAAAGCGCTCTGAAACCTC	56

15 (281bp)	15F: 5’- TGCTGAGGATGCCTCTGTCT 15R:5’- GCGACCCTTGTGTGGTAGA	56
16 (218bp)	16F: 5’- GTGTTCCCAGTCTGCCATTTC 16R:5’- TGTGTGGACACTGTGTGACG	56
17 (289bp)	17F: 5’- CCTGGGGACTCACACACTG 17R:5’- CCTCACTTCCCGGACCAC	56

C. Primers, PCR conditions for *AFG3L2* mutation screening

Exon10 (310bp)	5’- CCGATTTATTTCATTTCTTATTCAG 5’- GCCTGGACGACAGAGTCA	56
Exon14 (333bp)	5’- TTGTGATAGGCAGCTCAGTC 5’- CTTTGCAGGAGTGTAGCTTG	56
Exon15 (297bp)	5’- GTCTTCATCTGTAGTAGGATCTTCAA 5’- CGTGCAAATATGAATACATGAGG	56
Exon16 (478bp)	5’- GCTTCTGGCTCTGTGTTT 5’- AGCCAGAGAGAGGGAATTCTG	56

Table S2 – Detailed genotypes of patients and familial history

Family	Code	Gender	Genotype	Mutation 1	Mutation 2	Familiar History	Age at onset
A	ATA-1	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	Brother of pt.2	58
A	ATA-2	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	Brother of pt.1	50
B	ATA-3	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	-	46
C	ATA-4	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	-	58
D	ATA-5	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	Grandparents first cousins	63
E	ATA-6	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	Parents first cousins	49
F	ATA-7	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	Brother of pt.9	41
F	ATA-8	**F**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	Sister of pt.8	40
G	ATA-9	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	-	51
H	ATA-10	**M**	Homoz	c.1529C>T; p.A510V	c.1529C>T; p.A510V	-	50
I	ATA-11	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.1617delC; p.V540Cfs*52	-	43
J	ATA-12	**M**	Compuond Heteroz	c.1529C>T; p.A510V	c.2228T>C; p.I743T	-	53
K	ATA-13	**M**	Compuond Heteroz	c.1529C>T; p.A510V	c.2102A>C; p.H701P	-	44
L	ATA-14	**M**	Compuond Heteroz	c.1529C>T; p.A510V	c.287-1 G>C; ?	negative	50
M	ATA-15	**M**	Compuond Heteroz	c.1529C>T; p.A510V	c.1553-2delAG; ?	-	38
N	ATA-16	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.1972G>A; A658T	2 affected brothers	52
O	ATA-17	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.1972G>A; A658T	-	29
P	ATA-18	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.773_774delTG; p.V258Gfs*30	Sister of pt.18	36
P	ATA-19	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.773_774delTG; p.V258Gfs*30	Sister of pt.17	41
Q	ATA-20	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.1369C>T; p.R457*	1St Cousin of pt.20	34
Q	ATA-21	**M**	Compuond Heteroz	c.1529C>T; p.A510V	c.1369C>T; p.R457*	1St Cousin of pt.19	39
R	ATA-22	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.1A>G; p.M1V	-	35

S	ATA-23	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.1877T>C; M626T	-	42
T	ATA-24	**M**	Compuond Heteroz	c.1529C>T; p.A510V	c. 637C>T; R213*	-	44
U	ATA-25	**F**	Compuond Heteroz	c.1529C>T; p.A510V	c.2181G>A; p.A727A	-	41

Table S3 – Detailed clinical symptoms at first examination

Family	Code	Gender	Genotype	Age at first	Symptom at	Gait	SARA score	Gait	Stance	Speech	Finger-chase	Heel-shin	Hyperreflexia	EPR	Spasticity	Ocular	Cerebellar signs	Bladder	Sensibility	MRI
A	ATA-1	**M**	Homoz	60	G	PPA	7	3	2	0	0	1	+	+, BBil	+	PEO (L)	+/- dysmetry	-	-	Cerebellar vermian atrophy
A	ATA-2	**M**	Homoz	56	G	PPA	2	1	1	0	0	0	+	+, BBil	+	PEO (L), PTO	+/-	-	-	
B	ATA-3	**M**	Homoz	54	G	A	6	2	1	1	1	1	+	Indif	-	NYS	+/- UL	-	An LL *	Mild fronto-parietal and brainstem atrophy. Mild cerebellar emispheric atrophy. Mild vermian Atrophy. No alteration of white matter.
C	ATA-4	**M**	Homoz	60	G	A							-	-	-	-	+LL	-	-	Marked cerebellar and vermian atrophy
D	ATA-5	**M**	Homoz	65	G	SA	8	2	1	2	1	2	+LL	+, BBil	+/-	PEO (L)	+/- UL +LL	-	N	Cerebellar Atrophy (Vermian+HE). Mild truncal atrophy
E	ATA-6	**M**	Homoz	60	G	A	6.5	2	0	1	0,5	1	+LL	-	-	Jerky SP	+/- UL + LL	-	HLL	Slight supra and infratentorial atrophy
F	ATA-7	**M**	Homoz	38	G+D	A	6	2	1	1	1	1	-	-	-	-	+	-	-	Cerebellar Atrophy (Vermian+HE)
F	ATA-8	**F**	Homoz	35	G+D	SA	8	2	1	2	1	2	+	+	+/-	-	+	-	LL	Mild cerebellar emispheric atrophy
G	ATA-9	**M**	Homoz	51	G	SA	4	1	1	0	1	1	++	+	+ LL	-	+ TRUNK AND LL	-	-	Mild cerebellar Atrophy
H	ATA-10	**M**	Homoz	74	G	SP	9	5	1	0	1	2	+	+	+	PEO SAC	+	U	LL	White matter hyperintensities leukoencephalopathy

I	ATA-11	**F**	Compound Heteroz	66	G	SP2	12	5	4	0	1	2	+	+	+	PEO (L, V), Sac	+/- UL + LL	-	-	Marked vermian and bilat emispheric atrophy , WM lesions
J	ATA-12	**M**	Compound Heteroz	55	G+R	SA	5	2	2	0	0	1	+LL	Indif	+/-	Sac hypometric	+/- UL +/- LL	-	N	White Matter lesion.
K	ATA-13	**M**	Compound Heteroz	48	G	A1	14	3	2	2	1	2	-	-	-	NYS	+/- UL + LL	-	-	Marked vermian and bilat emispheric atrophy
L	ATA-14	**M**	Compound Heteroz	51	G	A1	5	2	1	0	1	1	-	-	-	-	+LL	-	-	Cortical cerebellar atrophy
M	ATA-15	**M**	Compound Heteroz	49	G	A2	14	2	3	3	1	2	+	+	-	-	+/- UL + LL	-	-	Cerebellar vermian atrophy
N	ATA-16	**F**	Compound Heteroz	58	G	SA	4	1	1	0	0	1	+LL	-	+LL	-	slight + UL LL	-	HLL	Mild cerebellar and cortical atrophy
O	ATA-17	**F**	Compound Heteroz	48	G	SP	12	3	3	2	2	2	+	+	+	-	+	U	LL	Cortical Atrophy
P	ATA-18	**F**	Compound Heteroz	55	G+D	SP	14	4	3	2	2	3	+	+	+	+	+	-	LL	Cerebellar vermian atrophy
P	ATA-19	**F**	Compound Heteroz	53	G+D	SA	13	4	3	1	2	3	+	+	+	-	+	-	LL	Cerebellar vermian atrophy
Q	ATA-20	**F**	Compound Heteroz	40	D	SA	9	2	1	2	2	2	-	+	-	-	+	-	LL	Cerebellar atrophy
Q	ATA-21	**M**	Compound Heteroz	41	D	SA	8	2	1	2	2	1	+	+	-	-	+	-	UL, LL	Cerebellar atrophy
R	ATA-22	**F**	Compound Heteroz	43	R	PPA	10	3	2	1	2	2	+	+	++	-	-	-	-	Normal
S	ATA-23	**F**	Compound Heteroz	45	G+D	SA	9	2	2	2	1	2	-	+	+	-	+	-	-	Mild cerebellar emispheric atrophy

T	ATA-24	**M**	Compound Heteroz	50	G+R	PPA	11	3	3	1	2	2	+	+	++	-	-	U	LL	Cerebellar vermian atrophy
U	ATA-25	**F**	Compound Heteroz	46	G	PPA	5	2	1	0	1	1	+	+	+	-	+	-	LL	Normal

Abbreviations: G: impairment of gait; D: Dysartria; R: Rigidity
SP: spastic-paraparesis, SA: spastic ataxia, PPA: paraparetoataxia, A: ataxia; 1: without aid, 2: with cane, 3: wheel-chair
UL: Upper Limbs; LL: Lower Limbs
EPR: Extension Plantar Response; BBil: Babinski Bilateral; Indif: Indifferent
+/- mild; PEO: opthalmoparesis; Sac: slow saccades; PTO: ptosis; NYS: Nystagmus L lateral gaze, V: vertical gaze
I: incontinence; L: limb; U: urgency; N: normal; H: hypopallesthesia, An: anapallesthesia
NA Not Available

Table S4 – Detailed clinical symptoms at follow up.

Family	Code	Gender	Genotype	Duration of	Follow up (yrs)	Gait	SARA score	Gait	Stance	Speech	Finger-chase	Heel-shin	Hyperreflexia	EPR	Spasticity	Ocular	Cerebellar signs	Bladder	Sensibility	MRI
A	ATA-1	**M**	Homoz	12	10	SA	13.5	6	4	1	0	2.5	+	+	+	PEO, PTO	-	U	An	
A	ATA-2	**M**	Homoz	10	10	PPA	7	3	1	1	0	2	+	+	+	PEO, PTO	+/-	-		
B	ATA-3	**M**	Homoz	15	10	SA	10	3	2	2	1	2	+	+; BBil (2013)	+	PEO (L) (2009), Slow Sac	+/- ULLL	U	An LL	Cervical and dorsal Spinal cord atrophy. Vermian atrophy. Peduncolo cerebellare assottigliato.
C	ATA-4	**M**	Homoz	19		SA										-	+LL	-	-	Marked cerebellar and vermian atrophy
D	ATA-5	**M**	Homoz	7	5	SA 1	10.5	3	1	3	1	2.5	+LL	+	+/-	PEO (L), Sac hypometric	+LL	U	N	Cerebellar Atrophy (Vermian +HE)
E	ATA-6	**M**	Homoz	21	11	A	10	2	1	3	1	1	N	-	-	jerky SP	+/- UL, + LL	-	HLL	MRI: mild cerebellar and cortical atrophy
F	ATA-7	**M**	Homoz	13	2	SA	7	2	1	1	2	1	+	+	+/-	-	+	-	-	NA
F	ATA-8	**F**	Homoz	9	2	SA	11	3	1	2	2	3	++	+				-	-	NA
G	ATA-9	**M**	Homoz	15	5	SA	6.5	1	1	1	1.5	2	++	+	+					

H	ATA-10	**M**	Homoz	24	0.5	PPA	9	5	1	0	1	2	++	+	+	PEO SAC	+	U	UL LL	NA
I	ATA-11	**F**	Compound Heteroz	31	8	PPA3	14	6	5	0	1	2	+	+	+	PEO (V, L), Sac	+/- UL, LL	U, I	H	
J	ATA-12	**M**	Compound Heteroz	9	7	SA1	8	3	2	1	0	2	++ LL	+	+ +	PTO, PEO (L), sac, NYS	+/-, UL +LL	U	N	WM lesion; Mild Cerebellar Atrophy (V+H)
K	ATA-13	**M**	Compound Heteroz	17	13	SA3	31	7	6	4	2	3	+	+	+	PEO (V, L), Sac	UL, LL	-	An LL	Marked vermian and bilat emispheric atrophy
L	ATA-14	**M**	Compound Heteroz	19	10	SA	6	3	2	0	1	0	-	+	-	right-beat NYS	+/- UL	U, I	H	vermian and bilat emispheric cerebellar atrophy
M	ATA-15	**M**	Compound Heteroz	12	1	A2	NA	NA	NA	NA	NA	NA	+	+	-	-	+/- UL, + LL	-	-	cerebellar vermian atrophy
N	ATA-16	**F**	Compound Heteroz	9	3	SA	4	1	1	0	0	1	+ LL UL	-	+	-	slight + UL LL	-	HLL	NA
O	ATA-17	**F**	Compound Heteroz		1	SP	12	3	3	2	2	2	+	+	+	-	+ UL	U	-	NA
P	ATA-18	**F**	Compound Heteroz	19																
P	ATA-19	**F**	Compound Heteroz	12																
Q	ATA-20	**F**	Compound Heteroz	7	2	SA	10	2	2	2	2	2	+	+	+/-	-	+	-	LL	NA

Q	ATA-21	**M**	Compound Heteroz	2	0.5	SA	8	2	1	2	2	1	+	+	+	-	+	-	UL, LL	NA
R	ATA-22	**F**	Compound Heteroz	6	1	PPA	11	3	3	1	2	2	+	+	++	-	-	-	-	NA
S	ATA-23	**F**	Compound Heteroz	1	1	SA	10	2	2	2	2	2	+	+	+	-	+	-	-	NA
T	ATA-24	**M**	Compound Heteroz	10	3	SP	13	4	3	1	2	3	+	+	++	-	+ UL, LL	U	UL, LL	NA
U	ATA-25	**F**	Compound Heteroz	10	6	SP	8	3	2	1	1	1	++	+	+	-	+ UL, LL	U	UL, LL	vermian atrophy

Abbreviations: G: impairment of gait; D: Dysartria; R: Rigidity
SP: spastic-paraparesis, SA: spastic ataxia, PPA: paraparetoataxia, A: ataxia; 1: without aid, 2: with cane, 3: wheel-chair
UL: Upper Limbs; LL: Lower Limbs
EPR: Extension Plantar Response; BBil: Babinski Bilateral; Indif: Indifferent
+/- mild; PEO: opthalmoparesis; Sac: slow saccades; PTO: ptosis; NYS: Nystagmus L lateral gaze, V: vertical gaze
I: incontinence; L: limb; U: urgency; N: normal; H: hypopallesthesia, An: anapallesthesia
NA Not Available