Adult-onset autosomal recessive ataxia associated with neuronal ceroid lipofuscinosis type 5 gene (CLN5) mutations.

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ABSTRACT

Autosomal recessive inherited ataxias are a growing group of genetic disorders. We report two Italian siblings presenting in their mid-50s with difficulty in walking, dysarthria and progressive cognitive decline. Visual loss, ascribed to glaucoma, manifested a few years before the other symptoms. Brain MRI showed severe cerebellar atrophy, prevalent in the vermis, with marked cortical atrophy of both hemispheres. Exome sequencing identified a novel homozygous mutation (c.935G>A;p.Ser312Asn) in the ceroid neuronal lipofuscinosis type 5 gene (*CLN5*). Bioinformatics predictions and *in vitro* studies showed that the mutation was deleterious and likely affects ER-lysosome protein trafficking. Our findings support *CLN5* hypomorphic mutations cause autosomal recessive cerebellar ataxia, confirming other reports showing *CLN* mutations are associated with adult-onset neurodegenerative disorders. We suggest *CLN* genes should be considered in the molecular analyses of patients presenting with adult-onset autosomal recessive cerebellar ataxia.

INTRODUCTION

The hereditary ataxias are a highly genetically heterogeneous group of disorders phenotypically characterized by gait ataxia, incoordination of eye movements, speech, and hand movements, and usually associated with cerebellar atrophy. Autosomal dominant forms typically have adult-onset; conversely autosomal recessive ataxias usually have onset in childhood [10]. Clinically and neuroradiologically these latter diseases overlap with milder forms of Neuronal Ceroid Lipofuscinosis (NCLs), a group of progressive neurodegenerative disorders characterized by the intralysosomal accumulation in both neural and peripheral tissues of autofluorescent, electron-dense cytoplasmic lipopigments (bearing close resemblance to lipofuscin)[1]. Clinically, NCLs are characterized by a variable combination of visual impairment, cerebellar ataxia, drug-resistant progressive myoclonic epilepsy, behavioral disturbances, mental deterioration, and early death [17]. NCLs are mainly transmitted with an autosomal recessive inheritance, though rare autosomal dominantly inherited forms have been described [11, 13, 23], and largely occur in infancy or preteen ages.

At present, fourteen NCL forms are recognized, with 13 disease genes (named *CLN*) identified and more than 400 mutations reported (NCL Mutation Database: www.ucl.ac.uk/ncl/mutation).

Here we describe an autosomal recessive form of adult-onset cerebellar ataxia associated with a novel *CLN5* missense change in two siblings.

MATERIALS AND METHODS

We studied ATA-7-TO family originating from the North-West of Italy, in which the two affected sibling were born from first cousins parents. Genomic DNA was extracted from peripheral blood (Qiagen, Hilden, Germany) following the manufacturer instructions. Molecular testing was negative for SCA1, SCA2, and Friedreich Ataxia. Exome sequencing and data analysis was performed implementing a previously described procedure [9] (see supplement).

Validation of the mutation was performed by Sanger sequencing in all members of the family with

primers designed to the exon 4 region of *CLN5* (Reference sequence NM_006493, NP_006484.1). Informed consent was obtained from all participants, . The study is a retrospective case report that does not require ethics committee approval.

Human wild-type CLN5–pCMV (a.a. 1–407, CLN5-wt) and CLN5–pCMV carrying the Finnish major disease causing mutation p.Tyr392* (CLN5-fin) have previously been described [16]. The CLN5-Ser312Asn vector was generated by *in vitro* mutagenesis, using the Quick Change site directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

HEK293 cells were grown overnight on coverslips coated with gelatin 0.1% in 24-well chambers and cultivated in DMEM 10%FCS at 37°C and in presence of 5% CO_2. Transient transfection with vectors containing CLN5-wt, CLN5-fin or CLN5-Ser312Asn cDNA was performed with Turbofect reagent (Thermo Fisher Scientific, Asheville, NC, USA) using the reported protocol [16] (see supplement).

RESULTS

The 55-year-old proband (III-6 in figure 1A) was a woman who presented with gait instability, dysarthria, and a mild cognitive deficit. Her past medical history had been unremarkable until she first sought neurological expert consultation after age 50 because of unsteady gait. Neurologic examination at age 58 was significant for a slight attention deficit, modest nystagmus with vertical component, dysarthric slurred speech, mild head tremor, and truncal ataxia. We also observed mild dysmetria and dysdiadochokinesia, an ataxic gait and posture, normal sensation, and brisk deep reflexes in the four limbs with normal plantar flexor response. Somatosensory evoked potentials, electroencephalogram (EEG), and nerve conduction velocities were all normal, as well as routine blood tests (including blood count, total cholesterol, alpha-fetoprotein and vitamin E levels) .

Brain CT scan and MRI at age 58 showed a marked cerebellar atrophy (more evident in the vermis than the hemispheres) with a severe bilateral cortical atrophy - mainly parietal and temporal - without focal lesions (Figure 1B, C).

At age 52, three years before recognition of her neurological symptoms, the patient complained visual problems that were initially attributed to glaucoma. Ophthalmological examination at age 58 revealed a limited visual field in both eyes with a paracentral scotoma in the left eye. Fundoscopy disclosed a bilateral pale and excavated optic disc consistent with a severe galucoma but we could not exclude a previous lesion of the optic nerve.

At the age of 61 years, the patient showed a worsening of her dysarthria, could not walk without help, and showed a severe intentional tremor in the four limbs. Upon appropriate testing, including an age/education-adjusted Mini Mental State Examination (MMSE) score of 19.4/30 , and a Montreal Cognitive Assessment score of 10/30, we also documented a more severe cognitive impairment with weakness especially in visual/spatial and executive tests, language, abstract symbolic reasoning and action planning. Conversely, time and space orientation, short term memory and calculation were relatively preserved.

Her elder brother (patient III-5 in figure 1A) was first examined when he was 61 years old. He presented with gait problems and dysarthria since age 56 with a slight worsening over the past five years. Neurological evaluation showed an ataxic-spastic gait, nystagmus in all directions, dysarthria with slurred speech, marked dysmetria in the lower limbs, and brisk and symmetric deep reflexes in the limbs. Muscle strength and tone, as well as sensation, were not affected. A mild cognitive deficit was also evident at clinical examination, but the patient refused further neuropsychological testing. As for her sister, case III-6, also had glaucoma. Brain MRI was not available.

We performed whole exome sequencing on III-5 and III-6 (71x and 68x coverage depth, respectively; sequencing statistics in Table S1). Assuming an autosomal recessive model of inheritance, we identified a single homozygous variant c.935G>A on chr13:77574815 in the *CLN5* gene, changing a Ser312 to Asn (NM_006493, OMIM *608102). This mutation was confirmed by Sanger sequencing (Figure 1D), in homozygosis in both affected siblings and in heterozygosis in

the unaffected brother III-4.

Serine 312 is a highly evolutionary conserved amino acid in vertebrates and it change was predicted to be deleterious using different bioinformatics tools (Table S2, figure S1). We evaluated the consequences of the p.Ser312Asn on CLN5 subcellular localization using immunofluorescence microscopy after HEK293 transfection (Figure 1E-G). Wild-type protein localized to lysosomal vesicles as expected [16], whereas both the CLN5-Ser312Asn and the CLN5-fin (Finnish mutation used as positive control) were retained in the Endoplasmic Reticulum (ER) compartment. Using cycloheximide, an inhibitor of cytoplasmic protein synthesis, we showed the complete disappearing of the fluorescence signal in cells carrying the CLN5-Ser312Asn indicating an intrinsic instability of the mutated proteins (Figure 1H-J).

DISCUSSION

Approximately 5% of the *CLN* mutations have been associated with adult-onset neurological phenotypes (from 17 to 43 yrs) divided into three different subtypes: Kufs diseases, characterized by progressive myoclonus epilepsy and cognitive decline (Type A), or behavioral anomalies, dementia, motor dysfunction, ataxia and extrapyramidal and suprabulbar signs (Type B) [4, 18]; adult NCLs which include autosomal dominant Parry disease due to *DNJC5* mutations [13] and the recessive NCL type 11 associated with mutations in the progranulin gene [7](Table 1).

Ataxia is the first symptom in adult-onset Neuronal Ceroid Lipofuscinosis, often followed by rapid cognitive deterioration, epilepsy and retinal degeneration.

Using exome sequencing, we identified a homozygous c.935G>A (p.Ser312Asn) mutation in *CLN5* in two adult siblings initially presenting ataxia. Both bioinformatics analysis and functional studies in HEK293 cells support the pathogenetic role of the mutation. While the wild-type CLN5 co-localize with lysosomes [16], the p.Ser312Asn mutant protein was retained in the ER and did not reach the lysosome, in analogy with p.Tyr392*, a previously well characterized *CLN5* protein change causing an infantile-onset NCL in Finnish population. Therefore, our data further sustain the

hypothesis that *CLN5* mutations, including the one identified, impact the ER-lysosomal trafficking [15, 16].

Remarkably, both our cases had the onset of their neurological manifestations after 50 yrs in the form of cerebellar ataxia with mild cognitive impairment, and represent the most late-onset cases associated with *CLN* mutations. Although features of glaucoma in both patients might be related to other processes, including aging, it is tempting to assume that ocular manifestations were part of the neurodegenerative process [24]. Most *CLN5* mutations result in a classical disease with onset between 4-7 yrs, and few mutations are also associated with juvenile to early-adult onset. Three cases were described with onset at 17 yrs, carrying respectively the p.Tyr258Asp homozygous mutation [8], or the compound heterozygous p.Cys126Tyr / p.Tyr374Cys or p.Thr303CysfsX10 / p.Tyr374Cys mutations [24]. *CLN5* disease-causing mutations are spread through the gene, and include missense, splicing and nonsense alterations (www.ucl.ac.uk/ncl/mutation). We hypothesize that the amino acid position and/or the chemical-physical change seen in our patients behaves as hypomorphic because it has a less severe impact on the protein function as compared to the other CLN5 variants.

TPP1, whose canonical mutations cause NCL2, has also been recently associated with autosomal recessive ataxia SCAR7 [20], suggesting the existence of a novel category of adult-onset diseases associated with *CLN* mutations, presenting with cerebellar ataxia and progressive cognitive decline. These data indicate that *CLN2* and *CLN5* may be suspected in case of adult-onset autosomal recessive cerebellar ataxia, and foresees the identification of further *CLN* involved in adult-onset phenotypes.

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CONFLICT OF INTEREST: Yulan Chen and Hao Zhang are employees of BGI-Shenzhen. No further financial disclosure.

ETHICAL STANDARDS

This study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Written informed consent was obtained from the patient and approved by a local ethics committee.

FIGURE LEGENDS

Figure 1. Pedigree, brain MRI, mutation and cellular analyses.

A. Pedigree of family ATA-7-TO suggested an autosomal recessive transmission due to parents consanguinity. B and C. Axial and sagittal T1-weighted brain resonance magnetic images in patient II-5 at 58 yrs. A marked cerebellar hemispheric and a more severe vermian atrophy is present. Supratentorial atrophy is also evident. D Mutation c.935G>A; p.Ser312Asn, affects a highly conserved amino acid (interspecies protein alignment is reported in figure S1). Panels E-J, localization of the p.Ser312Asn *CLN5* mutant protein in HEK293 transfected cells. E-G. HEK293 cells transfected with human wild-type *CLN5*, CLN5-fin and CLN5-Ser312Asn mutants respectively. Immunostaining using rabbit antibody against CLN5 (1RmI-4) shows a diffuse punctate patter for wild-type protein consistent with its known lysosomal localization [16] (panel E). Both mutants show a diffuse reticular staining, compatible with a ER retention (panels F and G). Panels H-J illustrate the effect of cycloheximide treatment on CLN5 protein turnover (green). After three hours incubation with cycloheximide wild-type CLN5 maintains a lysosomal localization, while CLN5-fin and CLN5-Ser312Asn mutants completely disappear from ER, as demonstrated by counter-staining with the ER marker PDI (in red).

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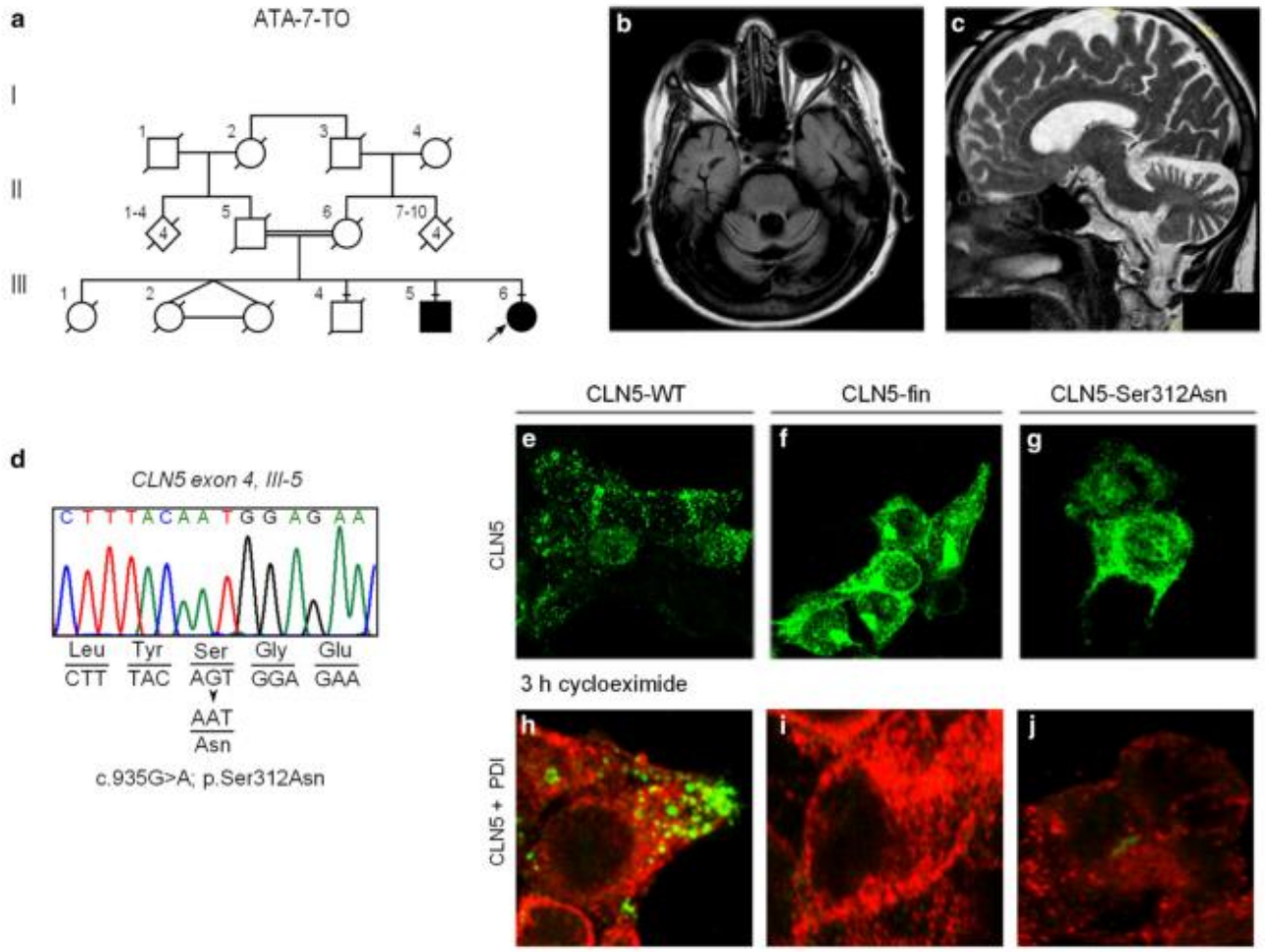
Table 1. Adult onset forms of neuronal Ceroid Lipofuscinosis

NCL category	GENE	Inheri-tance	Mutation	Number of Cases	Age at onset (yrs)	Phenotype at onset	Ref.
ANCL	*CLN1 (PPT)*	AR	p.Arg151* p.Gly108Arg	2	31-38	Psychiatric symptoms followed by ataxia, visual, verbal, and cognitive losses	[21]
	CLN1 (PPT)	AR	p.Arg151* p.Cys45Tyr	1	24	n.a.	[14]
	CLN4a (DNAJC5)	AD	p.Leu115Arg	1	34	Seizure, progressive dementia	[13]
	CLN4b (DNAJC5)	AD	p.Leu116del	1	25	Behavioural anomalies, seizure dementia, speech regression followed by ataxia	[13]
	CLN5	AR	p.Cys126Tyr p.Tyr374Cys	1	17	Cognitive regression and visual loss followed by seizure and motor difficulty	[24]
	CLN5	AR	p.Tyr303Cysfs*10 p.Tyr374Cys	1	17	Motor difficulty followed by seizure, visual loss and cognitive regression	[5]
	CLN6	AR	p.Tyr93Met p.Leu128Val	1	n.a.	n.a.	[12]
	CLN11 (GRN)	AR	p.Thr272Serfs*10	2	22-23	Visual failure and convulsion followed by ataxia	[19]
Type A KD	*CLN4a (DNAJC5)*	AD	p.Leu116del	1	26	Behavioural anomalies, followed by seizures	[22]
	CLN4a (DNAJC5)	AD	p.Leu116del	19	26-40	Myoclonus, Speech deterioration, Ataxia, Dementia	[6]
	CLN4a (DNAJC5)	AD	p.Leu115Arg	1	43	Myoclonus, Speech deterioration, Ataxia, Dementia	[6]

	CLN4a (DNAJC5)	AD	p.Leu116del	10	28-33	Myoclonus, Seizures, cognitive deterioration followed by motor impairment	[13]
	CLN6	AR	p.Leu47Phe	1	28	Myoclonus followed by ataxia and dementia. No visual loss.	[2]
	CLN6	AR	p.Arg6Thr	1	31	Seizures followed by myoclonus and dementia. No visual loss.	[2]
	CLN6	AR	p.Leu67Pro p.Arg103Gln	2	16-26	Myoclonus followed by seizures and dementia. No ataxia nor visual loss.	[2]
	CLN6	AR	p.Phe238Thr	3	17-51	Myclonus, ataxia and cognitive decline	[2]
	CLN6	AR	p.Tyr50* p.Asn77Lys p.Ser308Thr	1	n.a.	n.a.	[2]
	CLN6	AR	p.Arg149His p.Pro297Leufs*53	2	35-43	Seizure or ataxia followed by tremor and dementia	[2]
Type B KD	*CLN4b (DNAJC5)*	AD	p.Leu115Arg	1	25	Behavioral anomalies, dementia	[3]
	CLN6	AD	p.Ala34Thr	1	37	Dementia followed by seizures	[2]
	CLN13 (CTSF)	AR	p.Gln321Arg	1	24	Seizures. Vision preserved	[18]
	CLN13 (CTSF)	AR	p.Gly458Ala p.Ser480Leu	2	22-32	Progressive cerebellar syndrome, ataxia, dysarthria. Vision preserved	[18]
	CLN13 (CTSF)	AR	p.Tyr231Cys p.Ser319Leufs*27	1	35	Cognitive decline, mild dysarthria, mild gait ataxia with tremor.	[18]

Figure 1.


a
ATA-7-TO
I
II
III
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CLN5 exon 4, III-5
C T T T A C A A T G G A G A A
Leu CTT
Tyr TAC
Ser AGT
AAT
Asn
Gly GGA
Glu GAA
c.935G>A; p.Ser312Asn
CLN5-WT
CLN5-fin
CLN5-Ser312Asn
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3 h cycloeximide
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CLN5 + PDI

Supplement

Materials and methods

Exome sequencing data analysis

Briefly, we captured exonic regions from genomic DNA by using Agilent SureSelect Human All Exon kit, and performed pair-end sequencing on an Illumina HiSeq 2000. We followed two independent analysis workflows to perform sequencing read alignment, variant calling and variant annotation. In the first pipeline (P1), we employed Burrows-Wheeler alignment to map fastq files to the human reference genome (UCSC hg19)[11]. We called variants using Genome Analysis Tool Kit (GATK, version 1.4) [12] followed by functional annotation with Annovar [13] and SnpEff [14]. In the second pipeline (P2), we conducted alignment to UCSC hg19 by Short Oligonucleotide Analysis Package (SOAP, version2.21) [15], then used SOAPsnp (version 1.05) [16] to identify single nucleotide variants (SNVs) as well as GATK [12] to detect small insertion-deletions (indels). We annotated variants by BGI's self-developed scripts in the second pipeline.

We assumed an autosomal recessive inheritance model, and filtered variants generated in each pipeline, as described in supplement.

First we retained homozygous mutations shared by both affected individuals, and excluded variants that meet any of the following criteria: 1) intronic regions >20bp from exon boundaries; 2) synonymous changes; 3) minor allele frequency (MAF) > 0.5% in one of the following databases: dbSNP135 (http://www.ncbi.nlm.nih.gov/snp), 1000 Genomes Project (n = 1,092 genotyped samples) (www.1000genomes.org), HapMap Project (n = 1,301 genotyped samples) (http://hapmap.ncbi.nlm.nih.gov), NHLBI Exome Sequencing Project (n = 6,500 exomes) (https://esp.gs.washington.edu/drupal/), CAG-CHOP (n = 669 exomes), or BGI internal controls (n = 1,414). We particularly considered variants close to splice sites and frameshift indels. Next, we prioritized the resulting variant list according to evolutionary conservation and filtered out regions with PhyloP [18] value<0.95; then retained variants predicted as "deleterious/damaging" by PolyPheN [19] and SIFT [20]. At last, we considered biological and clinical relevance of cerebellar

ataxia for the identified candidates.

Immunofluorescence

Forty-eight hours after transfection, cells were fixed in ice-cold methanol for 3 min and then blocked with 0.5% bovine serum albumin (BSA) in phosphate buffered-saline (PBS) for 30 minutes. In the cycloeximide experiment, cells were treated with 50µg/ml cycloheximide (Sigma-Aldrich, St. Louis, Missouri, USA) to stop protein synthesis for three hours prior to fixing. For co-localization studies we used primary antibodies against PDI for ER staining (1:200) (Sigma-Aldrich, St. Louis, Missouri, USA). CLN5 –antibody (1RmI-4) was used with dilutions previously reported in [21]. Primary and secondary antibodies were diluted in blocking buffer and incubated for 1 hr respectively at 37°C or room temperature. Stained coverslips were visualized using Olympus confocal microscope (Olympus, Center Valley, PA, 18034, USA).

Table S1. Sequencing summary statistics for the samples, as well as variant numbers called by CAG and BGI pipelines P1 and P2, respectively. Qualified variants mean those fall in exonic regions or at splicing donor/recipient sites.

Sample	Family relation ship	Total reads mapped to genome (Mb)	Total data on target region (Gb)	Mean depth of target region	Coverage of target region (%)	Total SNV's called (P1/P2)	Qualified SNV's called (P1/P2)	Total indel's called (P1/P2)	Qualifie d indel's called (P1/P2)
III-5	Affected brother	41.67	3.15	68.50	98.82	39,981/79,214	16,366/17,5 67	6,388/5,926	620/697
III-6	Affected sister	43.38	3.26	71.11	98.98	44,979/84,423	16,682/17,9 80	7,060/6,237	630/718

Table S2: *In silico* prediction of the p.Ser312Arg mutation on CLN5 protein

Software	Score	Predicted effect	Web site
SIFT	0	Deleterious	http://sift.bii.a-star.edu.sg/
Mutation T@ster	46	Disease causing	www.mutationtaster.org/
MAPP	0.001664	Bad	http://mendel.stanford.edu/sidowlab/downloads/MAPP/index.html
Polyphen 2	1.0	Probably Damaging	www.genetics.bwh.harvard.edu/pph2/
I-MUTANT	2	Reduced Stability	http://folding.biofold.org/i-mutant/i-mutant2.0.html

Figure S1. Evolutionary conservation of Serine 312 in vertebrates