ORIGINAL ARTICLE

Giant axonal neuropathy: a multicenter retrospective study with genotypic spectrum expansion

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Abstract
Giant axonal neuropathy (GAN) is an autosomal recessive disease caused by mutations in the *GAN* gene encoding gigaxonin. Patients develop a progressive sensorimotor neuropathy affecting peripheral nervous system (PNS) and central nervous system (CNS). Methods: In this multicenter observational retrospective study, we recorded French patients with *GAN* mutations, and 10 patients were identified. Mean age of patients was 9.7 years (2–18), eight patients were female (80%), and all patients met infant developmental milestones and had a family history of consanguinity. Mean age at disease onset was 3.3 years (1–5), and progressive cerebellar ataxia and distal motor weakness were the initial symptoms in all cases. Proximal motor weakness and bulbar symptoms appeared at a mean age of 12 years (8–14), and patients used a wheelchair at a mean age of 16 years (14–18). One patient died at age 18 years from aspiration pneumonia. In all cases, nerve conduction studies showed a mixed demyelinating and axonal sensorimotor neuropathy and MRI showed brain and cerebellum white matter abnormalities. Polyneuropathy and encephalopathy both aggravated during the course of the disease. Patients also showed a variety of associated findings, including curly hair (100% of cases), pes cavus (80%), ophthalmic abnormalities (30%), and scoliosis (30%). Five new *GAN* mutations were found, including the first synonymous mutation and a large intragenic deletion. Our findings expand the genotypic spectrum of *GAN* mutations, with relevant implications for molecular analysis of this gene, and confirm that GAN is an age-related progressive neurodegenerative disease involving PNS and CNS.

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Introduction

Giant axonal neuropathy (GAN, OMIM #256850), is an autosomal recessive progressive disease affecting the peripheral and central nervous systems (PNS and CNS). "Kinky hair" (tightly curled hair not observed in parents) and long eyelashes are characteristic features of the disease, albeit not observed in all cases [1]. GAN usually presents as a childhood-onset axonal neuropathy, with a majority of patients losing ambulation in their teens and loosing life in their thirties [1]. CNS involvement may include leukoencephalopathy, optic atrophy, nystagmus, cerebellar dysfunction, intellectual disability, and spasticity [1]. Nerve biopsy shows reduced density of nerve fibers and large axons with neurofilament accumulation [1].

The gene causing GAN was identified in the year 2000 and more than 80 different *GAN* mutations have been described in approximately 100 patients since then [2–12]. *GAN* encodes the 65-kDa protein gigaxonin, a BTB/Kelch protein with an N-terminal BTB (Bric-a-brac, Tramtrack and Broad) domain, central BACK domain, and 6 Kelch repeats [1]. There is no clustering of mutations, and a majority of patients present with point mutations in the BTB, BACK, and 6 Kelch repeat domains [1]. It was suggested that GAN was an E3 ligase substrate adaptor possibly involved in the regulation of intermediate filaments (IFs) turnover [1]. To date, there is no treatment for GAN.

In this multicenter observational retrospective study, we report the detailed molecular, electrophysiological, brain magnetic resonance imaging (MRI), and clinical characteristics of 10 patients with 5 new *GAN* mutations, including 1 patient with a large intragenic deletion and 2 patients with a synonymous mutation.

Patients and methods

Protocol approvals, patients' consents, and registration

Patients

We performed an observational retrospective study including French patients with pathogenic homozygous variations in the *GAN* gene. Patients were identified from French laboratories sequencing the *GAN* gene (Lyon, Paris), and data collection was performed in hospitals following the patients. Six pediatricians and neurologists were contacted, and clinical, paraclinical, and genetic data were compiled from medical records by the referring physician. Data gathering was performed between January 2018 and September 2018.

Electrophysiological studies

Electrodiagnostic studies were performed in 7 patients including upper and lower limbs nerve conduction studies (NCS) and electromyography (EMG) using a concentric needle electrode in at least 2 muscles. Studies were achieved using regular equipment and accepted methods, and skin temperature was kept in 32°–34 °C range.

Additional tests

All patients underwent pulmonary function tests (PFT) and standardized biological tests. Brain MRI was performed in 6 patients. Vocal cord mobility was investigated using laryngoscopy in 3 patients with dysphonia. Muscle biopsy was performed in 1 patient and processed with standard methods for histology and histochemistry.

Genetic analysis

The 11 exon and intron junctions of the *GAN* gene were sequenced by Sanger di-deoxynucleotide method in all patients. Numbering of mutations was done using RefSeq cDNA NM_022489.3. All patients gave informed consent for genetic analysis.

In silico splicing prediction

No cDNA sample was available in family F2, and we used 4 splice site algorithms to predict a potential splicing effect: MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSplice (http://www.fruitfly.org/), GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml), and Human Splicing Finder (http://www.umd.be/HSF/) [13–15].

Minigene constructs & in vitro splicing test

Minigene constructions for splicing studies were performed as previously described using the (CTAAACAGCCACATATGACGGATGCCTCAGATCTGGG) and (CCCCCCTCGACCATATGGGCAGGATAAATGTGAAGGC) primers [16]. The sequence we analyzed included the end of *GAN* intron 5 (400 nucleotides), the entire exon 6 (113 nucleotides), and the beginning of intron 6 (243 nucleotides). Human HeLa cells were used for triplicate transfections [16].

Results

Patients

We found 10 patients from 5 distinct families (Table 1). Mean age of patients was 9.7 years (2–18), eight patients were female (80%), and all patients met infant developmental milestones and had a family history of consanguinity (Table 2). Mean age at disease onset was 3.3 years (1–5), and progressive cerebellar ataxia and distal motor weakness were the initial symptoms in all cases (Fig. 1). Proximal motor weakness and bulbar symptoms, i.e., dysphonia and dysphagia, appeared at a mean age of 12 years (8–14), and patients used a wheelchair at a mean age of 16 years (14–18) (Fig. 2). One patient died at age 18 of aspiration pneumonia. Patients also showed an assortment of associated findings, including curly hair (100% of cases), pes cavus (80%), ophthalmic abnormalities (30%), scoliosis (30%), tendon contractures (20%), hirsutism (20%), precocious puberty (10%), vocal cord paralysis (10%), and hearing impairment (10%) (Table 2).

Electrophysiological studies

NCS studies showed abnormal upper & lower limb compound muscle action potentials (CMAPs), sensory nerve action potential (SNAPs), and motor and sensory nerve conduction velocities (MSNCVs) in all 7 investigated cases (Supplemental Table 1). EMG demonstrated moderate neuropathic changes in distal lower limb muscles of all patients, and we found no pathological spontaneous activity. In our series, CMAPs, SNAPs, and MSNCVs alterations tend to aggravate with age, with sensory and motor responses progressively disappearing after 9 years of age (Supplemental Table 1). To summarize, our patients had a slowly progressive, symmetrical, predominantly distal, sensorimotor neuropathy with axonal and demyelinating features.

Additional tests

Brain MRI was abnormal in all 6 investigated cases (Fig. 3). Cerebellar abnormalities, including abnormal dentate nuclei and leukopathy, were observed in all cases (Fig. 3). Brain white matter abnormalities were observed in all cases, with optical radiations involved at an early stage, and internal capsule, periventricular and subcortical white matter, thalami, and brainstem involved at later stages (Fig. 3). Taken together, our data demonstrate that our patients had a slowly progressive diffuse leukoencephalopathy involving brain, cerebellum, and brainstem. Laryngoscopy showed vocal cord paralysis in 1 patient with dysphonia. In one case, tibialis anterior muscle biopsy found neurogenic abnormalities.

Genetic analysis and in silico splicing prediction

Five new homozygous mutations in the *GAN* gene were identified in the 5 families reported in our study, including missense mutations in the *GAN* Kelch domain in the *F*3 (Glu493Lys) and *F*4 (Arg458Trp) families (Fig. 4). *GAN* mutations segregated in families *F*1, *F*2, *F*4, and family DNA was not available in families *F*3 and *F*5. In family *F*1, exons 2 to 9 of GAN could not be amplified by PCR, and CGH array analysis confirmed the homozygous deletion of GAN exons 2–9 (Fig. 4). In family *F*2, a homozygous synonymous *GAN* variant c.1086G>A (p.Glu362=) was found close to the end of exon 6 (Fig. 4). In silico splicing analysis predicted an abolition of the donor site of exon 6 (Supplemental Table 2). In family *F*3, a rare *GAN* homozygous p.Glu493Lys (c.1477G>A) (rs780427229) variant was identified in exon 9 (Fig. 4). The frequency of this variant in gnomAD database was 0.0018% for non-Finnish Europeans, the affected amino acid was conserved in vertebrates, and bioinformatic tools predicted the variation to be deleterious (PolyPhen-2: probably damaging, score 0.999; SIFT: tolerated, score 0.26) (Supplemental Table 3). In family *F*4, a *GAN* homozygous p.Arg458Trp (c.1372A>T) variation was found at the end of exon 8 (Fig. 4). The mutation was absent in the 1000 Genomes, ExAC, and gnomAD databases, affected an amino-acid conserved from zebrafish to humans, and in silico predictions were consistent with a deleterious substitution (SIFT: deleterious, score 0; PolyPhen-2: probably damaging, score 1.000) (Supplemental Table 3). Codon 458 overlaps exons 8 and 9, and in silico analysis predicted a limited impact

Table 1 Families with *GAN* mutations identified in this study

Family (*F*)	Mutation	Amino acid change	Exon	Country of origin	Familial history	Consanguinity	Number affected
*F*1	exons 2–9 deletion	NA	2–9	Algeria	Autosomal recessive	+	1
*F*2	c.1086G>A	Glu362=	6	France	Autosomal recessive	+	2
*F*3	c.1477G>A	Glu493Lys	9	France	Autosomal recessive	+	3
*F*4	c.1372A>T	Arg458Trp	8	Algeria	Autosomal recessive	+	3
*F*5	c.155G>A	Ser52Asn	1	Algeria	Autosomal recessive	+	1

NA, not applicable

Table 2 Detailed clinical characteristics of 10 patients with *GAN* mutations

Patient, family (F)	Amino acid change	Sex	Age at onset (years)	Disease duration when examined (years)	Distal weakness	Proximal weakness	Absent tendon reflexes	Cereb ataxia	Dysphagia	Curly hair /ichtyosis	Loss of ambulation	Motor and sensory axonal neuropathy (NCS)	Brain white matter abnormalities (MRI)	Other features
1. F1	exons 2–9 del	F	4	12	LL+UL	LL+UL	+	+	+	+/+	+	ND	+	nyst, earl pub
2. F2	Glu362=	F	3	7	LL	-	+	+	-	+/-	-	+	ND	tend contract
3. F2	Glu362=	F	4	2	LL	-	+	+	-	+/-	-	+	ND	tend contract
4. F3	Glu493Lys	F	3	5	LL+UL	LL	+	+	+	+/+	-	+	+	nyst, hirsu
5. F3	Glu493Lys	F	3	15	LL+UL	LL+UL	+	+	+	+/+	+	+	+	hirsu, resp ins
6. F3	Glu493Lys	F	1	1	-	-	+	+	-	+/+	-	ND	ND	-
7. F4	Arg458Trp	M	5	4	-	-	+	+	-	+/-	-	+	+	-
8. F4	Arg458Trp	M	3	12	LL+UL	LL+UL	+	+	+	+/-	+	+	+	opt atr, resp ins
9. F4	Arg458Trp	F	4	4	-	-	+	+	-	+/-	-	ND	ND	-
10. F5	Ser52Asn	F	3	12	LL+UL	-	+	+	-	+/-	-	+	+	resp ins
Total	-	-	-	-	70%	40%	100%	100%	40%	100%	30%	100%	100%	-

Cereb, cerebellar; *Del*, deletion; *Earl pub*, early puberty; *Hirsu*, hirsutism; *LL*, lower limb; *MRI*, magnetic resonance imaging; *NCS*, nerve conduction studies; *ND*, not done; *Nyst*, nystagmus; *Opt atr*, optic atrophy; *Resp ins*, respiratory insufficiency; *Tend contract*, tendon contractures; *UL*, upper limb

Fig. 1 Clinical features of patients with GAN mutations **a** Patient 3, age 6 years: distal lower limbs atrophy (black arrows). **b** Patient 10, age 15 years: distal upper limbs atrophy (white arrows). **c** Patient 2, age 10 years: curly hair

on the donor site of exon 8 (mean value− 15 %). In family *F*5, a *GAN* homozygous p.Ser52Asn (c.155G>A) variation was found in exon 1 (Fig. 4). The variant was absent in the 1000 Genomes, ExAC, and gnomAD databases, and affected an amino-acid conserved from zebrafish to humans (Supplemental Table 3). To note, a mutation on the same codon (p.Ser52Gly) was previously reported in the patient with GAN (Bomont 2000).

In vitro splicing assay analysis

As no cDNA was available in family *F*2, minigene experiments were performed to investigate the molecular effects of
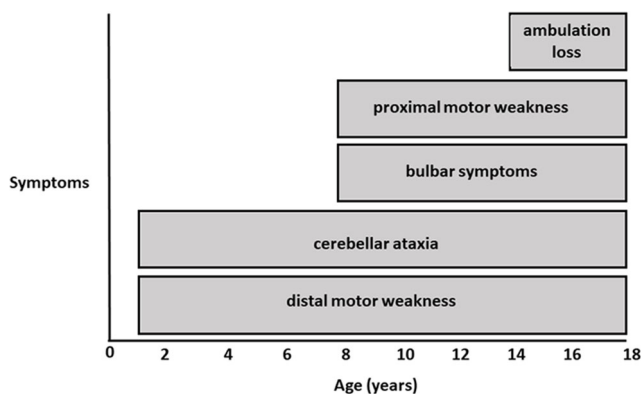

Fig. 2 Time of occurrence of the main clinical features Distal motor weakness appeared at a mean age of 2.9 years (1–5, *n* = 10); cerebellar ataxia at a mean age of 3.3 years (1–5; *n* = 10); bulbar symptoms at a mean age of 10.4 years (8–14; *n* = 4); proximal motor weakness at a mean age of 12 years (8–14; *n* = 3); and ambulation loss at a mean age of 16 years (14–18; *n* = 2). One patient died at age of 18 years.

the p.Glu362=variant. Minigene experiments revealed a reduction of the 360 bp normal *GAN* mRNA and the presence of an alternate 375 bp splicing product (Fig. 5a). Sequencing of the minigene product demonstrated that the first 15 nucleotides of *GAN* intron 6, which contain an in-frame stop codon, were retained (Fig. 5b). At protein level, retention of this intronic in-frame stop codon provokes the appearance of a premature stop codon at position 366 (Fig. 5b).

Discussion

In this multicenter observational retrospective study, we identified 10 patients with 5 new *GAN* mutations, including 1 patient with the first large intragenic *GAN* deletion (exons 2–9 deletion) and 2 patients with the first *GAN* synonymous mutation (p.Glu362=).

The phenotypic spectrum observed in our series of 10 patients was homogeneous, with progressive cerebellar ataxia and distal motor weakness appearing at age 1–5 years, later followed by proximal motor weakness and bulbar symptoms appearing at age 8–14 years, and eventually loss of ambulation appearing at age 14–16 years (Table 2, Figs. 1 and 2). Friedreich Ataxia Rating Scale & the Gross Motor Function measure, which have been recently validated to assess motor function in GAN, were not analyzed in our patients [17]. Importantly, all patients in our series met infant developmental milestones, had a family history of consanguinity, and presented with curly hair, the latter being in our series as an excellent clinical marker of the disease. However, not all GAN patients have curly hair, and it has been suggested that straight
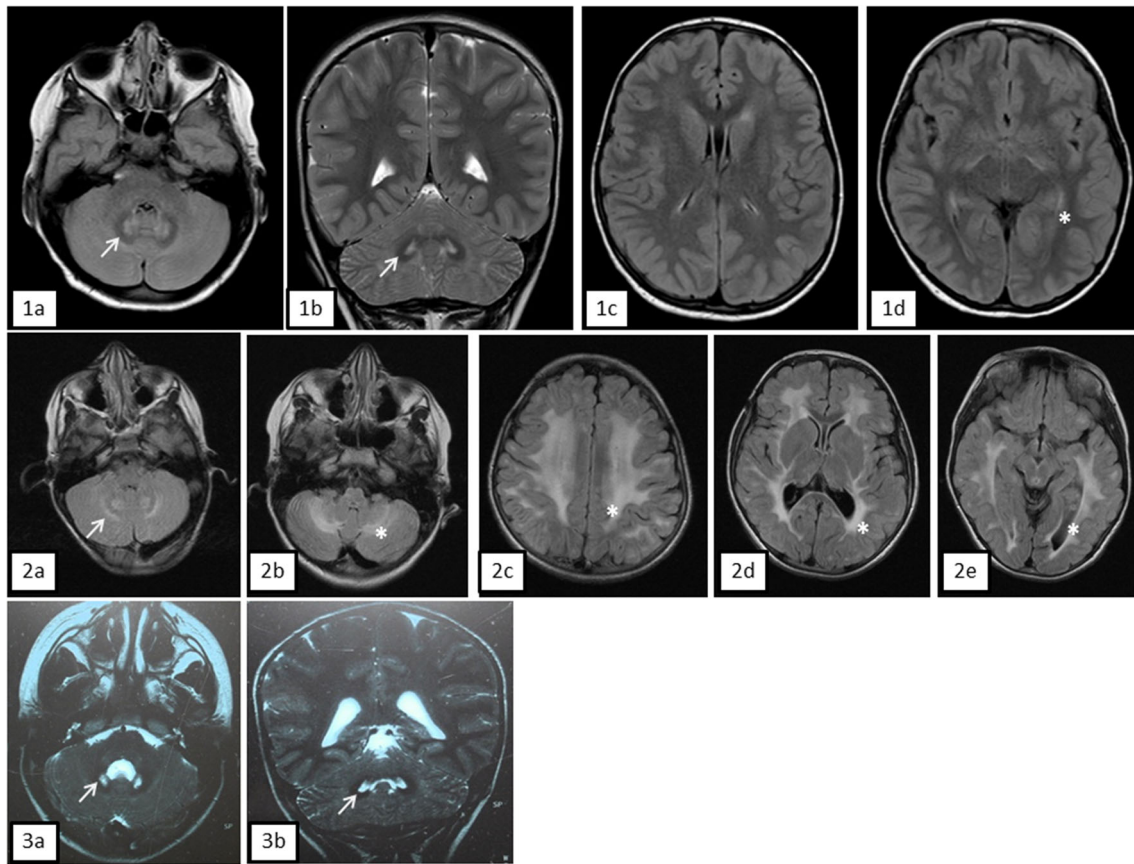
Fig. 3 Brain MRI of patients with *GAN* mutations Brain MRI of three patients with *GAN* mutations at age 6 years (1a–d), 7 years (2a–e), and 14 years (3a–b). MRI shows cerebellar abnormalities in all patients at all ages, including abnormal dentate nuclei with a central hyperintense signal surrounded by a hypointense signal observed in T2-FLAIR sequences (1a–b; 2a arrows) and T2-weighted sequences (3a–b; arrows), and cerebellar leukopathy (2b; asterisk). MRI also shows brain white matter abnormalities involving optical radiations at an early stage (1d; asterisk), and involving internal capsule, periventricular and subcortical white matter, thalami, and brainstem at an advanced stage (2c–e; asterisks).
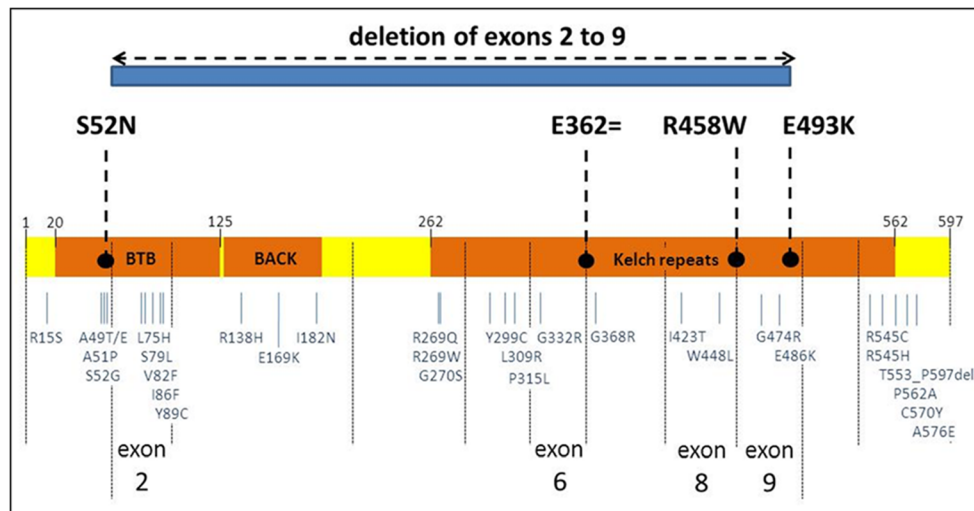

Fig. 4 *GAN* mutations reported in this study Schematic representation of GAN cDNA in yellow with functional domains in orange. The 5 new mutations described in this study (S52N, E362=, R458W, E493K, and exons 2–9 deletion) are on top of the figure, and their corresponding exons are delineated with dotted lines. Previously reported missense mutations are in gray (Johnson-Kerner BL et al., Muscle Nerve 2014; 50:467–76; Wang L et al., Muscle Nerve 2014; 50:200–5; Roth LA et al., Neuromuscl Disord 2014; 24 :48–55; Koichihara R et al., Brain Dev 2016; 38:350–3)

Fig. 5 In vitro splicing assay for the *GAN* c.1086G>A (p.Glu362=) mutation **a** The c.1086C>A mutation provokes a massive reduction of the 360 bp normal *GAN* mRNA and the appearance of an alternate 375 bp splicing product. **b** Sequencing of the minigene product demonstrates the retention of the first 15 nucleotides of *GAN* intron 6 which contain an in-frame stop codon. At protein level, the retention of this intronic in-frame stop codon provokes the appearance of a premature stop codon at position 366
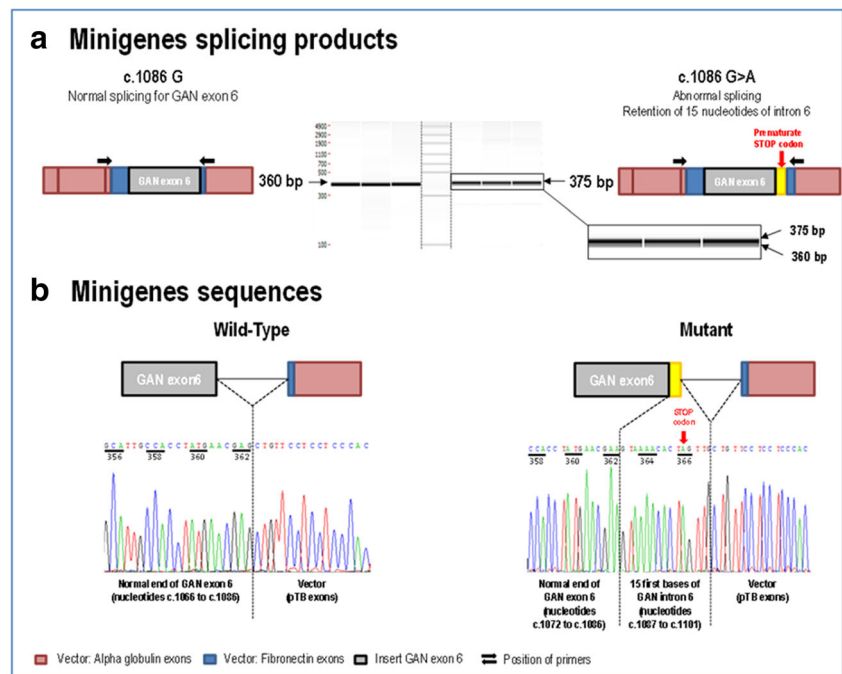
hair may be correlated with a phenotypically milder disease [1, 9]. More recently, the use of next generation sequencing (NGS), including exome sequencing and targeted gene panel analysis, has shown that *GAN* mutations can be associated in a few families with a typical intermediate or axonal Charcot-Marie-Tooth phenotype (CMT2) without any additional signs and symptoms [11, 18, 19]. Eventually, the clinical findings we noted are in accordance with other series demonstrating that *GAN* mutations provoke a progressive sensorimotor neuropathy affecting both PNS and CNS [1].

In our series, brain MRI was abnormal in all 6 investigated cases (Fig. 3). Interestingly, in contrast with cerebellar abnormalities which were observed in all cases at all ages, brain white matter abnormalities progressively spread with age, with optical radiations involved at an early stage, and internal capsule, periventricular and subcortical white matter, thalami, and brainstem involved at later stages (Fig. 3). Similar MRI abnormalities have been observed in several GAN cases, but the course of GAN CNS MRI abnormalities are hard to scrutinize because of the condition scarcity [1, 6]. Interestingly, our results suggest that *GAN* mutations provoke an age-related progressive leukoencephalopathy initially involving the cerebellum, and latter spreading to the brain hemispheres and brainstem (Fig. 3).

When considering electrodiagnostic studies, our data demonstrate that our patients presented with a slowly progressive, symmetrical, predominantly distal, axonal, and demyelinating sensorimotor neuropathy

(Supplemental Table 1). For the most part, these findings are in accordance with other published series [1]. Interestingly, in our series, CMAPs, SNAPs, and MSNCVs alterations tended to aggravate with age, with sensory and motor responses progressively disappearing after 9 years of age, suggesting age-related progressive axon loss (Supplemental Table 1).

In our study, we found 5 new deleterious *GAN* mutations in 5 families, including the first *GAN* synonymous mutation (p.Glu362=) and a large *GAN* large intragenic deletion (exons 2–9 deletion) (Fig. 4, Supplemental Table 2). To clarify the effect of the c.1086G>A (p.Glu362=) synonymous variant, we performed in vitro splicing assays which showed that this variation provokes the appearance of an in-frame cryptic premature stop codon in intron 6 (Fig. 5). The splicing assay confirmed the abolition of the exon 6 donor site predicted by the 4 prediction algorithms (Supplemental Table 3). At protein level, the synonymous p.Glu362= mutation is therefore most likely a frameshift mutation with a premature stop codon (p.Ala363Valfs*3). Overall, these results are in accordance with what has been previously shown in GAN, i.e., the absence of mutation clustering, the predominance of missense point mutations, and the scarcity of intragenic deletions [1, 20, 21].

GAN pathological studies usually show enlarged axons filled with compact, densely bundled IFs [1]. GAN postmortem studies usually show PNS and CNS neurodegeneration, including axon swellings and spheroids in the spinal cord, brainstem, and cerebral cortex

(reviewed in [1]). In GAN, a derangement of many IF classes is observed in neurons, astrocytes, Schwann cells, perineurial cells, endothelial cells, muscle fibers, fibroblasts, and lens epithelial cells [22–24]. Inclusion content is cell-type dependent and includes neurofilaments (NFs), peripherin, alpha-internexin, vimentin, glial fibrillary acidic protein, nestin, desmin, and keratin [22, 23, 25]. The exact role of GAN protein is unknown, although its role as an E3 ligase substrate adaptor involved in IF turnover regulation is strongly suspected [22, 23, 25]. There is no correlation between GAN protein level and disease severity, suggesting that the amount of gigaxonin needed in a cell has a critical value [22, 23, 25].

There is currently no approved therapy for GAN patients. However, it has been recently shown that an adeno-associated viral (AAV) vector bearing a normal copy of the human GAN transgene (AAV9/JeT-GAN) restored normal IFs configuration in patient fibroblasts and GAN knock-out (KO) mice [26]. Interestingly, intrathecal delivery of AAV9/JeT-GAN in GAN KO mice led to conserved sciatic nerve ultrastructure, lowered IF accumulations in neurons, alleviated rotarod dysfunction, and maintained wild-type PNS and CNS gigaxonin expression [24]. As a result, a phase I trial using viral-mediated GAN gene replacement is underway (https://clinicaltrials.gov/ct2/show/NCT02362438) [26].

Contributorship statement AEL, JMC, LGM, PA, LK, NB, AV, KB, JP, BF, and PL designed the research, performed the research, and participated in the data collection. Andoni Echaniz-Laguna and Philippe Latour wrote the manuscript. JMC, LGM, PA, LK, NB, AV, KB, JP, and BF revised the manuscript for intellectual content.

Competing interests The authors declare that they have no competing interests.

Compliance with ethical standards

This study was accepted and achieved under the ethical guidelines issued by our institutions for clinical studies. This study was in compliance with the Helsinki Declaration. All patients gave informed consent.

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