Progressive Ataxia Associated With Scarring Skin Lesions and Vertical Gaze Palsy

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Xeroderma pigmentosum (XP) is a rare autosomal-recessive disease (prevalence of 1:200,000 in whites) characterized by hypersensitivity to ultraviolet (UV) radiation because of faulty DNA repair, leading to early-onset recurrent sunburns with cutaneous hyperpigmentation and a 1000-fold increased likelihood of developing dermal and ocular neoplasias, commonly nonmelanoma skin cancer, followed by melanoma.[1,2] Seven genes (*XP A–G*) are known to be involved in the process of nucleotide excision repair of UV-induced DNA defects.[1] *XP V* is involved in the replication process of damaged DNA.[1] More than 20% of XP patients present with neurologic abnormalities, typically ataxia, hyporeflexia, bulbar symptoms, hearing impairment, and severe mental retardation.[2,3] These typically develop when the aforementioned skin abnormalities are already present.[2] Patients with neurologic symptoms have an earlier mean onset age of cutaneous symptoms (6 months) compared with patients without neurologic symptoms (2 years).[2] Although patients with neurologic abnormalities usually have a more severe cutaneous phenotype,[4] mean survival is similar in both groups, with only 5% of patients surviving beyond the age of 45.[2] Here, we describe 2 adult siblings of Turkish origin (pedigree shown in Fig. 1) with a complex neurological syndrome, distinct skin lesions, and a homozygous mutation in the *XP A* gene (c.682C>T; p.R228X).

Case Presentations

Patient 1

The index patient (Fig. 1), a 35-year-old man, developed stuttering at the age of 8. At age 12, he noticed gait difficulties with recurrent falls, clumsiness, and abrupt, involuntary movements. He also developed mental slowing, inattentiveness, and forgetfulness. He has 2 healthy children (ages 7 and 5). Over the last 5 years he became wheelchair bound, stopped speaking, and developed severe swallowing and hearing problems. Since childhood he has had recurrent facial sunburns.

On examination, the patient's skin at the nose was scarred from recurrent sunburns. His face was hyperpigmented. He had echopraxia, apraxia, and a tendency to perseverate. He had difficulties initiating internally generated saccades, with compensatory head thrust, saccadic hypometria, and supranuclear vertical gaze palsy (Video 1). Tendon reflexes were abolished in the legs. The Babinski sign was negative bilaterally. There was no muscle wasting but distal leg weakness. Sensory examination was not feasible. His speech was unintelligible. Residual vocalizations appeared both bulbar and cerebellar. Finger and hand movements were slow. There was mild intention tremor, dysmetria and marked dysdiadochokinesia in both arms. When extending the arms, there was a combination of mild dystonic posturing, chorea, and also athetoid arm movements, probably caused by impaired joint position sense (pseudoathetosis). He had prominent trunk instability. When trying to walk, he had both sensory ataxia and a stepping gait.

Supporting Information may be found in the online version of this article.

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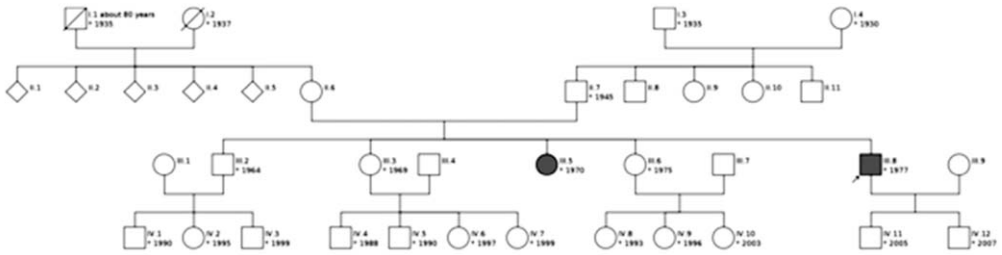

FIG. 1. Pedigree of the family reported. Affected siblings are marked with black symbols. The index patient is indicated by an arrow.

A cMRI showed microcephaly with supratentorial and cerebellar atrophy (Fig. 2A,B). Neurophysiologic examinations revealed severe axonal sensorimotor polyneuropathy and audiometry sensorineural hearing loss. There was no hepatosplenomegaly. The concentration of alpha-fetoprotein in plasma was normal (2.8 ng/mL). Previously performed sequencing of the *NPC1, NPC2*, and *Senataxin* genes excluded known disease-causing mutations.

Patient 2

The patient's 42-year-old sister (see pedigree in Fig. 1) also had remarkable sensitivity to sunlight, with recurrent burns since early childhood. Multiple skin excisions, predominantly from her face, had previously revealed basal-cell carcinomas. Since the age of 19 she had slowly developed problems similar to those of her brother, with severe cognitive decline, hearing difficulties, involuntary arm movements, and problems with coordination, speech, and swallowing. She was still able to walk with a Zimmer frame.

On examination, she appeared progeric and had multiple facial skin lesions and hyperpigmentation. She was demented and had a dysexecutive syndrome and apraxia. She had difficulties in generating internal saccades, saccadic hypometria, saccadic gaze pursuit, and marked hypometria of vertical saccades (Video 2). No square-wave jerks and no nystagmus were observed. Vestibulo-ocular reflex testing was normal. She had mild distal leg weakness without muscle wasting. Leg tendon reflexes could not be elicited. The Babinski sign was negative bilaterally. She had severe dysarthria with dysphonia. There was dysdiadochokinesia, mild intention tremor, mild generalized chorea, and some dystonic posturing and pseudoathetosis in her hands and arms. Gait was slow, shuffling, and mildly ataxic. Previous CCT scans had revealed microcephaly with global brain atrophy. An audiogram showed sensorineural hearing loss.

Because of the unavailability of a sequencing panel of all involved XP genes (*XP A–G* and *V*), we resorted to exome sequencing, which, by a massively high throughput approach, revealed a homozygous pathogenic mutation in the *XP A* gene (c.682C>T; p.R228X),[5] reproduced by Sanger sequencing and confirming the clinical diagnosis of XP.

Discussion

The bewildering spectrum of early-onset cerebellar ataxias often prompts specialists to meander through a plethora of differential diagnoses, which on clinical grounds alone are difficult to confirm or to refute. The

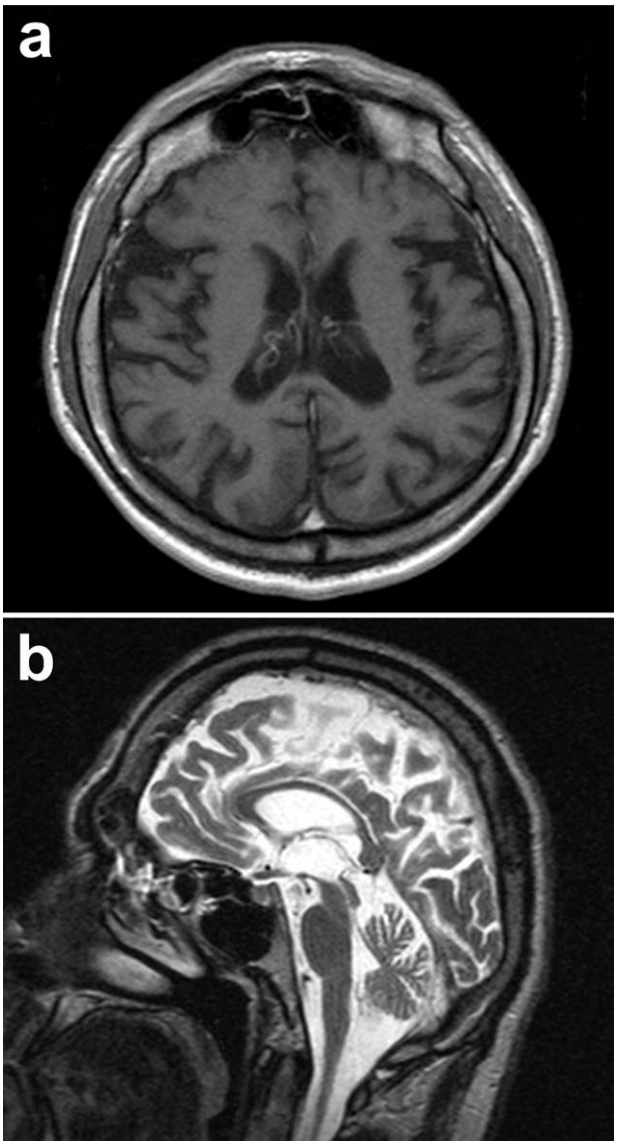

FIG. 2. Axial T1 (**A**) and sagittal T2 (**B**) MRIs show microcephaly and supratentorial and cerebellar atrophy.

A1

acknowledgment of certain age-dependent signs can be paramount in guiding the diagnostic route. In this familial case of a probable autosomal-recessive disease with onset in late childhood/early adolescence and a complex clinical presentation with saccadic initiation difficulties, supranuclear vertical gaze palsy, signs of mixed motor and sensory polyneuropathy with distal weakness, pseudoathetosis, and sensory ataxia, cerebellar ataxia, and mild dystonia, together with severe dysarthria, cognitive decline, sensorineural deafness, and microcephaly, the first diagnostic considerations would include lysosomal-storage diseases like Niemann-Pick type C (NPC), as well as ataxia telangiectasia (AT) and AT-like disorders (including ataxia with oculomotor apraxia types 1 and 2). Whereas additional clinical, laboratory, and imaging information (eg, presence of neuropathy, lack of hepatosplenomegaly, normal AFP levels) would potentially reduce the likelihood of NPC, AT, and ataxia with oculomotor apraxia type 2, the most striking sign in the presented cases was skin lesions (scars from sunburns, hyperpigmentation, and multiple basal-cell carcinomas, without telangiectasia).

To our knowledge, this is the first report of a classic XP-associated neurological syndrome with supranuclear vertical gaze palsy. However, whether this sign is unusual in these patients or has so far not received adequate attention remains unclear. We therefore suggest considering XP in patients presenting with a combination of signs of UV hypersensitivity, early-onset cerebellar ataxia, oculomotor abnormalities including supranuclear vertical gaze palsy, severe dysarthria, polyneuropathy, and dystonia.

Legend to the Videos

Video 1. The index patient (Fig. 1A), a 35-year-old man, is shown. He has saccadic hypometria, supranuclear vertical gaze palsy, saccadic smooth pursuit, and bulbocerebellar dysphonia. There is mild appendicular dystonia and chorea and also athetosis on arm extension. Hand and finger movements are slow and dysmetric. He also has apraxia. The glabellar reflex is not habituating. In the applause test he perseverates.

Video 2. The index patient's sister, a 42-year-old woman, is shown. She has saccadic initiation difficulties and compensatory head thrust, incomplete supranuclear vertical gaze palsy, and saccadic hypometria. There is hypophonia and dysarthria with predominant bulbocerebellar affection. Mild dystonic posturing, chorea, and appendicular athetosis can be appreciated during arm extension. There is mild intention tremor, dysmetria, and severe dysdiadochokinesia. Tendon reflexes are abolished. At rest, there are also choreic leg movements. Her gait is shuffling and ataxic, with a flat foot strike. ■

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Commentary for "Progressive Ataxia Associated With Scarring Skin Lesions and Vertical Gaze Palsy"

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In their clinical vignette, Ganos et al. describe 2 siblings who turned out to have Xeroderma pigmentosa on exome sequencing. These cases are interesting for 2 reasons. First, the clinical manifestation of vertical supranuclear gaze palsy, which was present here, is unusual for Xeroderma pigmentosa, although the other features were fairly characteristic, including the teenage or early adult onset, cognitive abnormality, cerebellar dysfunction, and, of course, the characteristic skin changes. Hence, Xeroderma pigmentosa should be added to the movement disorders practitioner's list of ataxic conditions with such eye

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Short communication

Dystonia with aphonia, slow horizontal saccades, epilepsy and photic myoclonus: A novel syndrome?

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ABSTRACT

Background: Dystonia with anarthria and/or aphonia is a rare syndromic association. Here we present two cases with slowly progressive, severe generalized dystonia and aphonia, slow horizontal saccades, epilepsy and photic myoclonus.
Methods: Detailed clinical data were collected over two decades in the female (index) patient and for nine years in her similarly affected son. Sanger sequencing followed by exome sequencing was performed.
Results: Both patients had leg onset generalized dystonia with gradual rostral spread including prominent facial and oro-mandibular involvement. The index patient was anarthric, her son aphonic. Both had saccadic slowing, more marked for the horizontal plane, and subclinical epileptic activity. The index patient also had photic myoclonus and a combined axonal and demyelinating neuropathy. Known genetic causes of similar syndromes were not identified.
Conclusion: These cases with caudo-rostrally spreading generalized dystonia with prominent facial and oro-mandibular involvement, severe speech impairment, marked slowing of horizontal saccades, and photic myoclonus likely represent a novel entity.

1. Main text

Prominent oro-laryngo-mandibular involvement in combined generalized dystonia [1] usually signifies a progressive neurodegenerative disease. In some of these syndromes prominent tongue protrusion has been noted and can guide diagnostic considerations [2]. However, reports on cases with generalized dystonia and anarthria (i.e. the inability to articulate) and/or aphonia (i.e. the inability to vocalize) without prominent tongue protrusion are scarce [3–10]. Here, we present a unique clinical syndrome of generalized dystonia with aphonia, slow horizontal saccades, epilepsy and photic myoclonus in a German woman and her son (pedigree shown in Fig. 1A), which, to our knowledge, has not been previously described in the literature.

2. Methods

2.1. Clinical

Clinical details have been gathered over a period of 19 years for the female patient and 9 years for her son. Detailed methodologies on neuropathological and genetic investigations are provided in the Supplementary material section. Informed consent was obtained for all examinations, including video recordings for publication and was in accordance with German law, the local ethics committee and the declaration of Helsinki.

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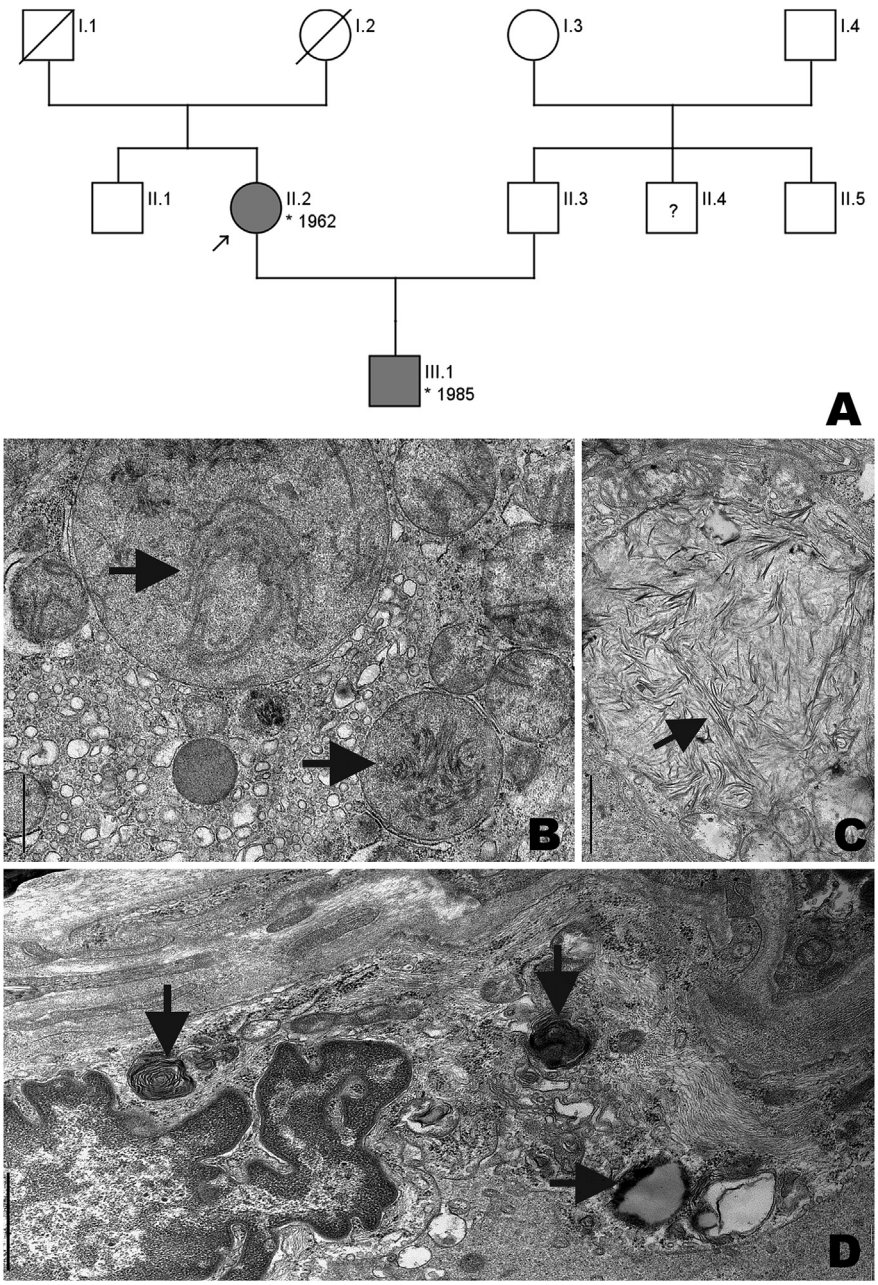

Fig. 1. A. Pedigree of the reported family. Affected members are marked with black symbols. The index patient is indicated by an arrow. Family member I1 (father of index patient) died at the age of 77 as a result of cardiac infarction and I2 (mother of index patient) at the age of 67 after suffering a stroke. Family member II4 was diagnosed with schizophrenia at late adolescence and died at his early thirties by an autoimmune disorder of unclear cause and is indicated with a question mark. B–D. Electron microscopic findings in skin biopsies of case 2. Large mitochondria with irregular cristae (B); Area with randomly scattered straight and curved filaments with a diameter of 18 nm in a cell of an eccrine gland in the axilla, same gland as in B (C); Myocyte of an arteriole with lysosomes and membrane bodies and mitochondria with irregular cristae (D); changes indicated with black arrows; scale bars = 1 μm.

3. Results

3.1. Clinical description

3.1.1. Case 1

Shortly after giving birth to her only son at the age of 23, this 51-year old woman developed gait difficulties with instability, inward feet rotation and difficulties reaching objects. She also developed pronounced problems with articulation and phonation. When suddenly exposed to light she reported to have brief generalized jerks.

On clinical examination (at the age of 51; video 1) her gingiva was hypertrophic. She had difficulties initiating saccades with compensatory eye blinking and marked slowing of saccadic velocity, more pronounced in the horizontal plane. Smooth pursuit was intact. There was no nystagmus. She had severe oromandibular dystonia with jaw opening dystonia. She was anarthric with only very limited phonation (short vocalizations). There was marked generalized

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A2

dystonia. Reflexes were preserved and plantar responses were flexor bilaterally. Sensory examination was unrevealing. Photic stimulation induced myoclonic jerks. There were no cerebellar signs. Although cognition appeared to be unaffected on clinical examination, motor disability precluded detailed neuropsychiatric or neuropsychological assessment. She appeared anxious.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2013.11.011.

Phoniatric evaluation showed slow tongue movements. There was reduced mobility of her right vocal cord. Chewing and swallowing were severely affected with dribbling and aspiration.

An MRI showed mild supratentorial and cerebellar atrophy. MR-spectroscopy was unrevealing. Neurophysiologic examinations showed axonal and demyelinating neuropathy of the peroneal and sural nerves. EEG revealed generalized epileptic activity. X-Ray examinations of her extremities and abdominal ultrasound were unremarkable. Full blood count, creatine kinase, acanthocytes, ceruloplasmin, alpha-fetoprotein levels, lysosomal enzymes (beta-glucosidase, acid sphingomyelinase, hexosaminidase A, beta-galactosidase) were normal. Neuropathological examinations of a muscle biopsy performed at the age of 37 demonstrated normal morphology on standard stains. Also SDH- and COX-activity were normal and there were no ragged red fibres. No accumulation of lipids or glycogen was shown within the muscle fibres. Activity of acid phosphatase was normal. Results from a skin biopsy taken at the age of 50 years also showed normal morphology on semithin sections. At the ultrastructural level, however, membrane bodies and mitochondria with rarefied cristae were present in some Schwann cells and myocytes of arterioles. No cytoplasmic inclusions or storage material was found.

3.1.2. Case 2

This 27-year-old man had delayed milestones (i.e. walking at the age of 2) and cognitive difficulties already apparent early in life. He attended a school for children with special needs where he learned reading and writing. At the age of 10 he developed gait difficulties with bilateral leg posturing. His speech at the time was unaffected. During adolescence symptoms spread rostrally to involve the entire body causing severe motor disabilities and affecting pharyngo-laryngeal, oromandibular and facial muscles giving rise to prominent speech and swallowing difficulties. His eye movements were noted to be abnormal. At the age of 23 he underwent pallidal deep brain stimulation surgery without any improvement.

Clinically, his tongue base was hypertrophic. He had severe generalized dystonia and aphonia (video 2). Oculomotor examination revealed prominent slowing of horizontal saccades. Smooth pursuit was normal. There was no nystagmus. There were no pyramidal signs. Reflexes were preserved and plantar responses were flexor bilaterally. There were no cerebellar signs and no photic myoclonus.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2013.11.011.

Phoniatric assessment demonstrated reduced tongue mobility. Flexible endoscopic evaluation of the pharyngo-laryngeal region revealed delayed initiation of chewing and swallowing. Although the mobility of the vocal cords was preserved, the patient was aphonic.

Previous investigations (acanthocyte counts, long-chained fatty acids, phytanic acid, lysosomal enzymes, ceruloplasmin, arginin, cholestanol, plasma amino acids, urine organic acids, cerebrospinal fluid neurometabolites) including cranial MRI, MR-spectroscopy and DaTscan were normal. EEG showed generalized epileptic activity. Two skin biopsies were taken at the ages of 26 and 27 years. In the first biopsy from the axilla some cells of the eccrine glands contained large atypical mitochondria with rarefied or condensed cristae (Fig. 1B). Areas of randomly orientated fibrils measuring 18 nm in diameter were observed occasionally in the cytoplasm of eccrine glands (Fig. 1C). In the second biopsy taken from the upper arm degenerative changes of mitochondria were noted in vascular myocytes and Schwann cells (Fig. 1D).

3.2. Genetic testing

Genetic testing for most common repeat expansion spinocerebellar ataxias (SCA1-3, 6, 7, 10, 12, 17, DRPLA), as well as Huntington's disease was normal. Due to the complexity of the disorder and the broad list of differential diagnoses of this novel phenotype we resorted to whole exome sequencing. Bioinformatic data analysis first focussed on excluding panels of genes known to cause dystonia, ataxia, myoclonus-epilepsies (Supplementary Table 1) and disorders of the dopamine-pathway (Supplementary Table 2A). In a next step all nuclear encoded mitochondrial genes were investigated (Supplementary Table 2B). No potential pathogenic variant could be identified. Candidate genes of secondary dystonic conditions with aphonia/anarthria (GM1, HEXA, HEXB, ATP7B, PANK2, FTL, HPRT1, NPC1, NPC2) were additionally screened. By conventional Sanger sequencing mutations in the mitochondrial genome were also excluded from blood, buccal swaps and urine sediment.

4. Discussion

In combined dystonia the recognition of additional signs can guide clinical reasoning [1]. In the presented cases, in addition to generalized dystonia, aphonia, horizontal saccadic slowing, epilepsy and photic myoclonus were clinical clues. However, well-recognized syndromes with dystonia and severe dysphonia/aphonia [3–10] were excluded. The index patient's son had a significant earlier onset (childhood onset compared to 23 years of age), which could either hint at an X linked inheritance, or anticipation or might just be explained by differences in severity of the disease. Slowing of horizontal saccades, epileptic activity and myoclonus are typical for the neuropathic form of Gaucher's disease, but this is an autosomal-recessive condition [11]. In addition, glucocerebrosidase activity levels were normal and genetic examinations were unrevealing. Although both patients had subclinical epileptic activity and the index patient additionally demonstrated photic myoclonus, the non-recessive inheritance pattern, and the lack of ataxic symptoms did not support the diagnosis of a progressive myoclonus epilepsy syndrome [12]. In addition, normal histology including normal enzymatic activities in a muscle biopsy of the index patient along with normal serum and CSF lactate were not in favour of a mitochondrial disorder. Also, mitochondrial genome sequencing (including nuclear encoded genes) was normal. However, degenerative alterations in mitochondria clearly exceeded physiological variations and could not be attributed to artefacts. Although mitochondrial degeneration may occur secondary to various conditions like chemotherapy, inflammation etc., this may indicate alterations of the oxidative metabolism in general or of the membrane structure/composition of the organelles [13].

As to the nature of aphonia, pseudobulbar and/or bulbar affection has to be considered. This is supported by slow tongue movements, hypomobility (i.e. paralysis) of the index patient's right vocal cord and difficulties in initiating chewing and swallowing. While one could argue that the aphonia might be part of dystonia, it is noteworthy that in most syndromes with prominent oro-laryngo-mandibular involvement severe dysarthria is the clinical hallmark and even in severe cases short words or syllables can be articulated. Furthermore, preserved facial expression of the two patients both for facial and oromandibular actions argues against aphonia being a consequence of apraxia.

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Prominent slowing of horizontal saccades and saccadic initiation difficulties are also in keeping with the assumption of brainstem impairment. Subclinical epileptic activity and photic myoclonus though indicate additional cerebral cortex involvement.

The long and seemingly static disease course, parallelled by the oral hypertrophic changes of both cases, the abnormal fibrillary cytoplasmatic depositions in eccrine glands observed in electron microscopy and the lack of nigrostriatal dopaminergic cell loss are in favour of metabolic/storage disorder.

To our knowledge, this is the first description of a severe, long lasting dystonic syndrome associated with aphonia, slow horizontal saccades, subclinical epileptic activity and photic myoclonus. We therefore believe this to be a novel entity. However, it might also represent an unknown phenotypic presentation of an established disorder, which our clinical, biochemical, neuropathological and genetic analyses did not identify. We therefore would like to raise clinicians' awareness for similar manifestations, which may be part of the spectrum of a new syndrome.

Author contributions

1. Drafting/revising the manuscript for content, including medical writing for content. 2. Acquisition of data. 3. Study supervision or coordination

CG: 1,2,3; SB: 1,2,3; SK: 1.2; AMO: 1,2; SH: 1,2; CH: 1,2; LS: 1, 3; KPB: 1,3; AM: 1,2,3.

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Appendix A. Supplementary data

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A2

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LETTER TO THE EDITOR

A distinct clinical phenotype in a German kindred with motor neuron disease carrying a *CHCHD10* mutation

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Sir,

Emerging data provide evidence for *CHCHD10* as a new candidate gene in familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Bannwarth *et al.*, 2014; Johnson *et al.*, 2014; Müller *et al.*, 2014). This gene encodes a mitochondrial protein located in the intermembrane space (Bannwarth *et al.*, 2014). Mutant *CHCHD10* may lead to altered mitochondrial genome stability and maintenance of cristae junctions (Bannwarth *et al.*, 2014; Chaussenot *et al.*, 2014). So far, three different mutations located either in the non-structured N-terminal region or in the α-helix of the *CHCHD10* gene have been attributed to cause both ALS and ALS-FTD phenotypes.

Here we report another large German family with a history suggestive of autosomal-dominant motor neuron disorder (Fig. 1). After excluding a repeat expansion in *C9orf72* and mutations in 25 other known ALS genes in parallel by next-generation sequencing we performed whole-exome sequencing of three affected individuals (Patients III.1, III.2 and III.9). This identified a heterozygous c.44G>T variant (p.Arg15Leu) in exon 2 of the *CHCHD10* gene which has recently been reported by Müller *et al.* (2014) as the likely cause of pure ALS in two German families and was also identified in three families with familial motor neuron disease in the USA (Johnson *et al.*, 2014). The mutation segregated with disease in another cousin (Patient III.5) of our index patient (Patient III.1) and could not be identified in his 41-year-old son (Patient IV.1) and an 85-year-old aunt (Patient II.7), who are both unaffected. No DNA samples were available from the deceased Patients I.1, II.3, II.6 and II.9, as well as from further to-date unaffected family members. However, because of the variable age of onset ranging from 41 to 73 years (59.5 $\pm$ 11.2 years; mean $\pm$ SD) only such individuals without clinical signs of a motor neuron disorder clearly after the latest disease onset within the family may really be regarded healthy. In our case, only individuals from the second generation (Fig. 1) would now have fulfilled this criterion with all other unaffected family members still being at risk.

Of note, seven of eight affected patients were males. All of them were diagnosed with motor neuron disease/ALS. Similar to the German families carrying the p.Arg15Leu mutation described by Müller *et al.* (2014), all of our patients exhibited upper limb onset exclusively, presenting with progressive, mostly atonic paresis, muscle wasting and fasciculations in either proximal (Patients II.6 and III.1 with symmetrical onset) or distal muscles (Patients III.2, III.5 and III.9 with a more asymmetrical distribution at onset), spreading out slowly and leading to severe disability of the upper extremities as disease progressed. At least three patients developed bulbar symptoms, however, not requiring supplemental tube feeding. None showed emotional instability. Patients III.1, III.2, III.5 and III.9

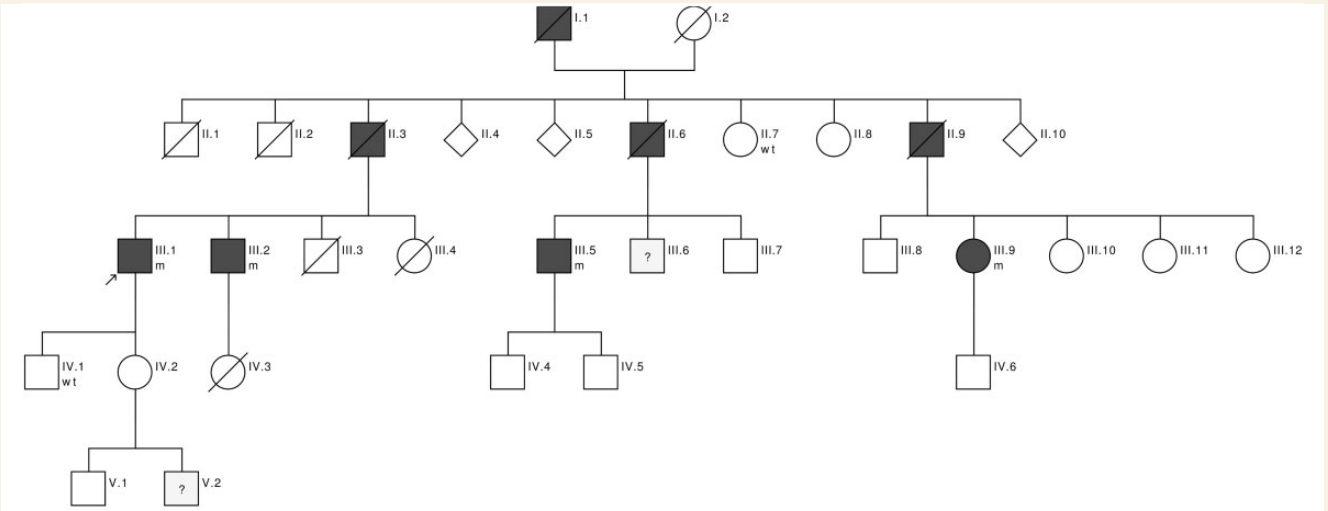

Figure 1 Kindred pedigree. Available genotypes are shown. The arrow denotes the index patient. Filled symbols represent affected individuals. DNA for genetic testing was not available from the deceased Patients I.1, II.3, II.6 and II.9, and from further unaffected individuals. Clinical and demographic information could not be obtained for all family members. Pedigree created with the CeGaT PedigreeChartDesigner. wt = wild-type; m = mutant.

have hyperreflexia and spasticity of the lower limbs and developed a spastic gait disorder, but not revealing marked weakness of the legs. Nevertheless, Patients III.5 and III.9 are wheelchair-bound due to imbalance as a result of the spastic gait and profound palsy of the upper extremities. After a disease course of 15 years, Patient III.5 has flaccid paraplegia of the arms and a pronounced dropped head syndrome, severe bulbar palsy as well as extensive hyperreflexia, spasticity and spontaneous cloni of both legs with largely preserved muscular strength. In the last follow-up, he exhibited cerebellar oculomotor disorder with abnormal smooth pursuits, dysmetric saccades and gaze-evoked nystagmus, but otherwise no signs of cerebellar ataxia. Patient III.6 presented with fasciculations but no further clinical and diagnostic signs of a motor neuron disease. However, he was lost to follow-up. Furthermore, one grandson (Patient V.2) of Patient III.1, who is now 12 years old, is suffering from muscle weakness and mental retardation of unknown aetiology since infancy. Work-up in a specialized genetic and neuropaediatric centre failed to establish a diagnosis. Symptoms do not seem to progress over time according to his mother, but to date he has not been available for clinical examination in our department. None of our patients have symptoms suggestive of frontotemporal lobar degeneration.

ALSFRS-R (ALS Functional Rating Scale, Revised; Cedarbaum *et al.*, 1999) score is available for four individuals ranging from 40 after a disease course of 2 years in Patient III.1 to 20 in Patient III.5 15 years after disease onset with a mean progression rate of 3.3 per year. EMG (performed in Patients III.1, III.2, III.5 and III.9) is indicative of a chronic neurogenic process rather than a myopathy. Neither of our patients had a muscle biopsy, but further examinations are in line with ALS diagnosis. Proton magnetic resonance spectroscopy was performed in Patients III.1, III.2 and III.5, and revealed neurodegeneration within the primary motor area and in the brainstem. Survival times of four patients already deceased (Patients I.1, II.3, II.6, II.9) range from 2 to 12 years (5.8 ± 4.5 years; mean $\pm$ SD). At present, four patients are still alive 2 to 15 years after disease onset (7 ± 5.6 years; mean $\pm$ SD). Patients with an early disease onset $\leqslant 50$ years had slower disease progression and survival times of >10 years as compared to family members who first became symptomatic in their seventies, suggesting that additional factors might contribute to a slowly progressive or else more aggressive phenotype. Unfortunately, no clinical information was provided by Johnson *et al.* (2014), but all patients were diagnosed with pure ALS.

In summary, we identified another ALS family with the heterozygous *CHCHD10* mutation c.44G>T (p.Arg15Leu). Thus far, among seven unrelated families with pure ALS and *CHCHD10* mutations, six have been reported to carry this variant. In contrast to the cases reported by Müller *et al.* (2014) our family history is suggestive of complete penetrance, given the absence of unaffected individuals transmitting the disease. However, we are aware that this conclusion has limited power due to the small number of unaffected individuals available for genetic testing. Lacking clinical details of the three families reported by Johnson *et al.* (2014) we still may hypothesize that the three German families share a common phenotype with upper limb onset and predominant lower motor neuron affection, spasticity and bulbar signs occurring later in the disease course and an overall slower disease progression, even though survival times reported by Müller *et al.* (2014) were somewhat longer than in our family. To date, cerebellar signs were only found in one

patient diagnosed with pure ALS, but that may also be due to other, e.g. vascular, reasons as he is a heavy smoker. However, a more aggressive phenotype may be associated with later disease onset.

Although functional studies for the p.Arg15Leu variant are lacking and determination of the frequency of *CHCHD10* mutations in larger cohorts and thus additional data on the phenotypic spectrum are needed, our data further substantiate the assumed causal genetic link between *CHCHD10* mutations and ALS. Perspectively, for a subset of patients with familial ALS it may even be a reasonable diagnostic algorithm to search for a *CHCHD10* mutation before screening other known ALS genes, if patients present with upper limb onset, lower motor neuron dominance, variable spasticity and bulbar signs occurring during the disease course, and a more or less slow to moderate clinical deterioration with sustained ability to walk and longer survival times up to more than 10 years. Nevertheless, even within a family there seems to be clinical variability, especially with regard to the age of onset and survival times.

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ORIGINAL COMMUNICATION

Clinical variability in ataxia–telangiectasia

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Abstract Ataxia–telangiectasia (A-T) is an autosomal recessive inherited disease characterized by progressive childhood-onset cerebellar ataxia, oculomotor apraxia, choreoathetosis and telangiectasias of the conjunctivae. Further symptoms may be immunodeficiency and frequent infections, and an increased risk of malignancy. As well as this classic manifestation, several other non-classic forms exist, including milder or incomplete A-T phenotypes caused by homozygous or compound heterozygous mutations in the ATM gene. Recently, ATM mutations have been found in 13 Canadian Mennonites with early-onset, isolated, predominantly cervical dystonia, in a French family with generalized dystonia and in an Indian family with dopa-responsive cervical dystonia. In this article, we will describe a Turkish family with three affected sibs. Their phenotypes range from pure cervical dystonia associated with hand tremor to truncal and more generalized dystonic postures. Exome sequencing has revealed the potentially pathogenic compound heterozygous variants p.V2716A and p.G301VfsX19 in the ATM gene. The variants segregated perfectly with the phenotypes within the family. Both mutations detected in ATM have been shown to be pathogenic, and the α-fetoprotein, a marker of ataxia telangiectasia, was found to be increased. This report supports recent literature showing that ATM mutations are not exclusively associated with A-T but may also cause a more, even intra-familial variable phenotype in particular in association with dystonia.

Keywords ATM · Dystonia · Turkey · Phenotype

Introduction

Ataxia–telangiectasia (A-T) is an autosomal recessive, complex, multisystem disorder characterized by progressive neurologic impairment, cerebellar ataxia [1], and variable immunodeficiency with susceptibility to sinopulmonary infections, impaired organ maturation, X-ray hypersensitivity, ocular and cutaneous telangiectasia, and a predisposition of malignancy.

Other phenotypes than classic A-T associated have been reported and these include adult-onset A-T, adult-onset spinal muscular atrophy, myoclonic jerks and hypotonia [2–4]. Recently, there is increasing evidence that different clinical presentations of dystonia are related to homozygous or compound heterozygous variants of the ATM gene [5–9].

Here, we report an additional family from Turkey carrying ATM gene mutations in a compound heterozygous state and we discuss the possible involvement of ATM in dystonia.

Electronic supplementary material The online version of this article (doi:10.1007/s00415-015-7762-z) contains supplementary material, which is available to authorized users.

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A4

Methods

Ethical approval for the study was obtained from the local ethics committee.

Patients

The participants are from Turkey, we have no report of parental consanguinity: the father hails from Sinop and the mother from Trabzon, both cities localised on the Black Sea coast of northern Turkey. The pedigree is shown in Fig. 1. The pattern of transmission is consistent with an autosomal recessive inheritance. The parents as well as three out of their six children were found to be neurologically normal.

Patient III:2 stood and walked uncertainly until the age of 4–5 years and had very slight slurred speech. Her symptoms did not progress and her further development was normal until the age of ~25 years when the patient presented a cervical dystonia. This was treated with injections of botulinum toxin. At the age of 35 years, she had a left side mastectomy after breast cancer. Only 1 year later the dystonic posture spread to the right upper limb. This was associated with head and hand tremor, and intermittent involuntary choreatic movements. The symptoms did not respond to levodopa or to clonazepam treatment. Additionally, the patient started to suffer migraine attacks and these were controlled by classical analgesics. Cerebral MRI was normal. At the age of 40 years, the patient underwent a total hysterectomy with bilateral salpingo-oophorectomy because of myoma and suspected cysts, but no additional pathogenic tumour was found.

Patient III:3 also developed very slight slurred speech and walked unsteadily at ages of 1–2 years after a short but severe episode of high fever. Symptoms were minor and

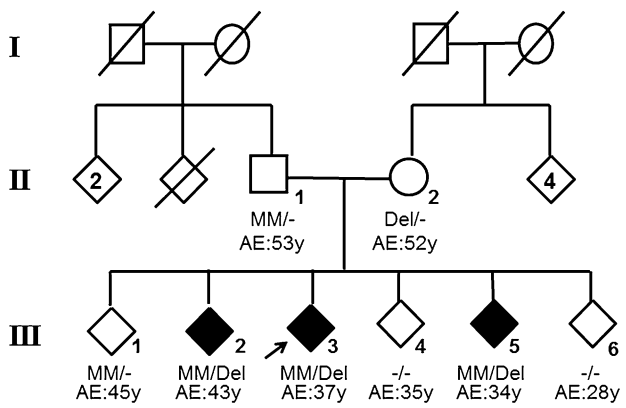

Fig. 1 Pedigree of the family. *MM* carrier of the p.V2716A ATM mutation, *Del* carrier of the c.1279 del a ATM mutation, *-/-* non-carrier, *AE* age at examination (years), *black filled symbols* affected patients

were not considered to be pathological until the age of about 30 years. At this time the patient started to suffer severe cramps and dystonic postures, particularly in the neck and right upper limb. Only a few months later he complained of involuntary choreoathetotic movements of the right hand. Analysis of the TORA1 gene was found to be normal. When the patient was examined for the first time in our department at age of 31, neurological examination showed a dysarthric slurred speech, a mild unsteady walk, a positive Babinski sign of the right foot and a manifest dystonic posture of the trunk and the right hand. All other examinations, such as a cerebral MRI and Tc-99m HMPAO-SPECT, examination of blood and urine, and testing for Wilson disease, including a liver biopsy, were normal. A therapeutic approach with levodopa did not benefit the patient, but his symptoms were clearly reduced by the anticholinergic biperiden. He was diagnosed with idiopathic dystonia. Subsequently, his symptoms progressed very slowly and at the age of 37, he also developed oculomotor apraxia and bilateral mild choreathetotic movements. A second cMRI was also normal and no ocular or cutaneous telangiectasia could be detected. Alphafoetoprotein, measured after the result of the molecular testing, was considerably elevated (142 IU/mL, reference 0–5.8).

The 34-year-old patient III:5 was interviewed by phone and then evaluated from a video. Since early childhood, she suffered from brief contractions, particularly in the upper limbs and the neck, but only rarely leading to an abnormal but painless posture. The patient also reported suffering of intermittent episodes of extension of her fingers in her right hand for 2 years. These would occur for instance when cutting vegetables, making the knife difficult to hold. The video sent by the patient shows a slight abnormal position of the head, with the neck marginally bent to the right side. She never received any treatment and had no particular medical history. She did not feel particularly handicapped in her daily life.

Videos of the patients are presented in the supplementary material.

After having obtained informed consent, genomic DNA was extracted from EDTA blood using a standard protocol.

Genetic testing

Exome sequencing

The coding and flanking intronic regions were enriched using the Agilent in solution technology and were sequenced using the Illumina HiSeq 2500 system. The resulting sequencing reads were mapped to the human reference genome (hg19) using the Burrows Wheeler Aligner (BWA-mem 0.7.2). Sequences, which did not

match a genomic position, were removed using Picard 1.14. Variants were called using SAMtools (v0.1.18) and VarScan (v2.3) and annotated based on the Ensembl database (v69). Only variants (SNVs/Small Indels) in the coding region and the flanking intronic regions (±8 bp) with a minor allele frequency (MAF) <1 % were evaluated. Minor allele frequencies were taken from the following databases: 1000 Genomes, dbSNP, NHLBI Exome Sequencing Project (ESP), and an in-house database. Sanger sequencing was performed to confirm the mutations found by WES in the probands and tested family members.

Quantitative real-time PCR

Large deletions and duplications affecting the SPR gene were excluded via quantitative real-time PCR using the Luminaris Color c ROX qPCR Master Mix (Thermo Scientific) and the QuantStudio 12 K Flex system (Life Technologies) according to the manufacturers' protocol for all coding exons.

Results

Screening of 40 genes (see supplementary material) known to be associated with dystonia by exome sequencing, revealed two mutations in the ATM gene in the index patient (III:3). We identified a single base pair deletion (p.G301V*fs*X19) in exon 7 which leads to a premature stop codon in exon 8 and the previously described pathogenic A-T missense mutation in exon 55 (p.V2716A) [10]. Both mutations did not exhibit any known frequency in the general population. Additionally one heterozygous missense variant p.V38I in the SPR gene was detected in patient III:3. Quantitative real-time PCR to detect a second mutation within the SPR gene however, was negative. The found ATM mutations co-segregated with the clinical symptoms in the family (Fig. 1a).

Discussion

We examined 10 members of a Turkish family and identified a compound heterozygous variants p.V2716A and p.G301V*fs*X19 in the ATM gene in all three of the affected family members presenting an uncommon phenotype of A-T. The spectrum of associated clinical signs in particular of movement disorders associated with A-T is known to be broad [3, 11]. Recently, reports of different types of dystonia related to ATM gene mutation attracted great attention [5–9]. The disease onset of classical A-T is generally in childhood or early adolescence, as seen in all three of the reported patients [1]. Interestingly, patient III:2 and patient III:3 started to show mild classical cerebellar symptoms, whereas the third sib described symptoms of paroxysmal dystonia. The disease then developed very differently in all three sibs. Instead of a rapid progression and disability at an early age, as usually described [12], the neurological phenotype was stable until age of 25 and 30 years in the patients III:2 and III:3, respectively, and did not develop any further in patient III:5. Subsequently, as previously described, the course of the disease is very individual.

Phenotypic variability, at least partially, has so far been explained by differences in the genotype: patients carrying truncating ATM mutations suffer a more severe phenotype than those with missense or site mutations. However, contrary to the common findings, we did detect major intra-familial variation in patients' disease, carrying all the same ATM genotype: one patient develops a phenotype in line with classical previously reported neurological symptoms, one patient develops cancer and different types of degenerated tissue and one patient is pretty stable only presenting mild symptoms of dystonia, and not feeling particularly handicapped in daily life. The reason for these great variations of clinical course might be provided by results of a very recent study: Verhagen and colleagues (2012) related the degree of ATM protein expression and kinase activity, to the different phenotypes [13]: Patients without ATM kinase activity showed classical symptoms, whereas residual kinase activity correlated with a milder and essentially different neurological phenotype and extended lifespan. The presence of ATM protein correlated with a slightly improved immunological function. However, we could not perform analysis of ATM protein expression and kinase activity within this family. We suggest that the individual allelic expression pattern of each patient might be responsible for the important differences of clinical symptoms: either the effect of the truncating mutation leading to incomplete and non-functional protein related to a more severe phenotype is dominant, or the allele carrying the missense mutation related to a less severe phenotype is predominant. ATM protein is expressed in most of the tissues (GeneCards) and depending on the predominance of the either deteriorated but still functional allele, or of the incomplete and non-functional protein, symptoms can vary. However, together with the molecular results of ATM analysis, protein expression and kinase activity might be very helpful tools in genetic counselling.

Our findings confirm previous findings that A-T is not only related to pure ataxia. The disease may appear as dystonia, especially of early onset, without frank cerebellar involvement and also normal cerebral imaging. A-T should be considered in all patients with unexplained, even mild movement disorders. Early diagnosis is important given the increased risk of malignancies, the related higher risk for

side effects of subsequent cancer treatment and for genetic counselling within particular large families.

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Conflicts of interest None.

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Serum Levels of Progranulin Do Not Reflect Cerebrospinal Fluid Levels in Neurodegenerative Disease

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Matthis Synofzik

Abstract: Altered progranulin levels play a major role in neurodegenerative diseases, like Alzheimer's dementia (AD), frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), even in the absence of *GRN* mutations. Increasing progranulin levels could hereby provide a novel treatment strategy. However, knowledge on progranulin regulation in neurodegenerative diseases remains limited. We here demonstrate that cerebrospinal fluid progranulin levels do not correlate with its serum levels in AD, FTD and ALS, indicating a differential regulation of its central and peripheral levels in neurodegeneration. Blood progranulin levels thus do not reliably predict central nervous progranulin levels and their response to future progranulin-increasing therapeutics.

Keywords: Alzheimer's dementia, amyotrophic lateral sclerosis, biomarker, case-control study, cerebrospinal fluid, frontotemporal dementia, *GRN*, progranulin, serum.

INTRODUCTION

Progranulin is a secreted protein which is expressed in multiple tissues and cell types throughout the human body, serving important roles in proliferation, inflammation and tumorigenesis [1]. In the brain, progranulin is implicated in both neuronal survival and neurodegenerative disease [2, 3]. The pathogenic role of altered progranulin levels hereby extends beyond genetic frontotemporal dementia with protein haploinsufficiency due to loss-of-function (LoF) mutations in the progranulin gene (*GRN)* [4, 5]. Rather, altered progranulin levels represent a universal theme shared across several common neurodegenerative diseases. In Alzheimer's disease, for instance, reduced brain levels of progranulin impair phagocytosis, increase plaque load and exacerbate cognitive deficits [6], while increased levels appear to protect against amyloid-β deposition and toxicity [6, 7]. Accordingly, increasing progranulin expression has been proposed as a novel treatment strategy in several neurodegenerative diseases [2, 6, 8]. However, although decisive for the understanding of neurodegenerative disease processes, knowledge on the regulation of progranulin levels is still limited. Given the specific contribution of progranulin to brain processes, we here hypothesised that central nervous system (CNS) progranulin levels in neurodegenerative disease are regulated differently from those in the body periphery. We therefore investigated the relation between cerebrospinal fluid (CSF) levels of progranulin and its serum levels in neurodegenerative diseases previously shown to be associated with progranulin alterations, namely frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS) and Alzheimer's dementia (AD).

MATERIALS AND METHODS

Ninety-six neurodegenerative patients, comprising of patients with FTD (n = 32, thereof 10 with behavioural variant FTD, 19 with progressive non-fluent aphasia and 3 with semantic dementia diagnosed according to established criteria [9, 10]), ALS (n = 35, diagnosed according to El Escorial Criteria [11]) and AD (n = 29, all diagnosed as clinically probable AD according to NINCDS-ADRDA criteria [12]), and 49 healthy controls were consecutively recruited from the Department of Neurodegenerative Disorders, University Hospital Tübingen. Control subjects did not show any signs of neurodegenerative disease, as ascertained by neurologists with special expertise in neurodegeneration, and underwent lumbar puncture for other reasons (e.g. disc prolapse; for subject characteristics see Table **1**). CSF and blood samples were taken within a time interval of max. 20 minutes. Bio-

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Table 1. Subject characteristics and progranulin levels.

Group	Controls	FTD	ALS	AD
subjects / thereof male	49 / 26 (53 %)	32 / 17 (53 %)	35 / 19 (54 %)	29 / 15 (52 %)
Age in years, mean (95 % CI)	63.61 (61.50 - 65.73)	66.72 (63.43 - 70.01)	63.51 (58.96 - 68.07)	68.62 (65.68 - 71.57)
Serum Progranulin in ng/ml, mean (95 % CI) [n]	212.86 (167.13 - 258.60) [24]	181.40 (143.68 – 219.31) [25]	189.09 (163.66 - 214.51) [26]	201.16 (176.85 - 225.48) [25]
Serum Progranulin-Albumin ratio in µg/g, mean (95 % CI) [n]	5.72 (4.09 - 7.35) [18]	4.62 (3.59 - 5.65) [25]	4.64 (3.84 - 5.44) [26]	4.90 (4.14 - 5.66) [24]
CSF Progranulin in ng/ml, mean (95 % CI) [n]	5.81 (5.26 - 6.37) [34]	4.73 (4.11 - 5.35) [28]	5.84 (5.19 - 6.50) [32]	5.02 (4.35 - 5.70) [28]
CSF Progranulin-Albumin ratio in µg/g, mean (95 % CI) [n]	23.95 (19.97 - 27.93) [30]	18.99 (14.74 - 23.24) [27]	24.58 (19.52 - 29.64) [32]	20.50 (17.56 - 23.44) [28]

Groups differed significantly in age (Kruskal-Wallis-test, χ^2 (3) = 8.296, p = .040, Bonferroni-corrected post-hoc tests not significant), but not in gender (Pearson Chi-Square-test, χ^2 (3) = 0.042, p = .998). AD, Alzheimer's dementia. ALS, amyotrophic lateral sclerosis. CI, confidence interval. CSF, cerebrospinal fluid. FTD, frontotemporal dementia. n, sample size.

material was stored at the local biobank (see Acknowledgements) and specimens were analysed without any previous thaw-freeze cycles. Measurements of progranulin levels were available for 100 serum samples (FTD: n = 25, ALS: n = 26, AD: n = 25, controls: n = 24) and 122 CSF samples (FTD: n = 28, ALS: n = 32, AD: n = 28, controls: n = 34). We performed the measurements by means of an established human progranulin ELISA (Adipogen, Liestal, Switzerland, for detailed ELISA characteristics, see Appendix **A**), which has been used for progranulin measurement in human plasma samples previously [13, 14]. Pairs of CSF and serum measurements were available in 91 subjects (FTD: n = 21, ALS: n = 23, AD: n = 25, controls: n = 22). Only these sample pairs were entered in the final correlation analysis. To identify LoF mutations in *GRN*, which are associated with substantially reduced blood progranulin levels and might thus confound the serum-CSF-correlations which are of interest here, we screened *GRN* in all subjects with serum progranulin levels below the established cut-off level of 110 ng/ml (sensitivity > 99 %, specificity > 92 % [13, 14]) by Sanger sequencing (n = 3) or exome sequencing (n = 2). We confirmed the specificity of our ELISA antibody by Westernblot analyses of CSF and serum samples (Appendix **D**). Sample collection, ELISA methods, Westernblot methods, genetic sequencing (whole exome sequencing/Sanger sequencing) and statistical analysis are described in more detail in Appendix **A**.

RESULTS

Correlations between Serum and CSF Progranulin

To test the hypothesis that CSF progranulin levels are regulated differently from serum progranulin levels, we estimated the partial correlation between subjects' CSF and serum progranulin levels in each group for all subjects of whom both CSF and serum samples were available (Figs. **1C, D, E, F**) (FTD: n = 21, ALS: n = 23, AD: n = 25, controls: n = 22). We hereby corrected for age and gender as covariates as these factors are known to influence progranulin regulation [15, 16]. Consistent with our hypothesis, CSF progranulin levels did not correlate with serum levels in any of the three neurodegenerative groups (FTD: r = 0.18, p = .235; ALS: r = 0.33, p = .075; AD: r = 0.10, p = .323), nor in controls (r = 0.16, p = .254, one-sided significance levels, partial correlation coefficients). Our quantitative ELISA measurements were compatible with the semi-quantitative Westernblot measurements which we obtained for CSF and serum samples of three subjects of each of the three neurodegenerative disease groups, selected for low, medium and high progranulin levels, respectively (Appendix **D**). To scrutinise our hypothesis further, we calculated the progranulin-albumin ratio in both CSF and serum and tested the correlation between subjects' CSF and serum progranulin-albumin ratios in each group (Appendix **C**). Again, no significant correlations were observed, neither in the neurodegenerative groups (FTD: r = 0.33, p = .071; ALS: r = 0.26, p = .120; AD: r = 0.22, p = .147), nor in controls (r = -0.09, p = .377, one-sided significance levels, Spearman's rank correlation coefficients).

Screening for *GRN* Loss-of-Function Mutations

As individuals with LoF *GRN* mutations show substantial reduction of progranulin levels due to haploinsufficiency [13, 14], inclusion of one or more of such cases could have biased the reported correlations. We therefore screened *GRN* in all subjects with a serum progranulin level below the es-

tablished cut-off level of 110 ng/ml (in total 5 subjects, thereof 4 FTD patients, 1 control, 0 ALS patients and 0 AD patients). This screening revealed two novel truncating *GRN* variants in 2 FTD patients (subject #19869: c.985_986insAC, p.D329fs; subject #13413: c.687T>G, p.Y229X; see Table **2** for details; both subjects are marked by filled circles in Fig. **1A, 1B** and **1D**). Also after excluding these two subjects from the group analysis, CSF progranulin levels in FTD patients did not correlate with serum levels (r = -0.23, p = .160).

DISCUSSION

Our findings indicate that CNS progranulin levels are regulated differently from peripheral progranulin levels in neurodegenerative disease. This notion is based on the missing correlation between the progranulin levels in these two compartments which could be observed in all three neurodegenerative groups investigated here. The absence of correlation held true upon correction for age and gender as covariates [15, 16] and upon exclusion of two FTD patients with likely pathogenic truncating *GRN* variants from the analysis whose serum progranulin levels were substantially reduced (subject #19869: 16.9 ng/ml; subject #13413: 1.5 ng/ml; see Table **2**). The absence of correlation is unlikely explained by the variation of progranulin levels over time [15, 17], since, in our study, CSF and blood samples were taken within a short time interval of max. 20 minutes. The absence of correlation is also unlikely explained by other physiological variations which may modify the total serum protein concentration (such as variations of subjects' nutritional state and hydration), since also the progranulin-albumin ratio did not yield any significant correlations between CSF and serum values. The absence of correlation was moreover unlikely due to interfering serum factors or unspecific binding of serum proteins by the ELISA antibody, since our Westernblot analyses demonstrated specific binding of the ELISA antibody in both CSF and serum (Appendix **D**). In line with these rather selective binding characteristics, this ELISA antibody has already been successfully used to measure plasma progranulin concentrations in previous landmark studies [13, 14]. Particularly, the ELISA antibody does not detect granulins or other progranulin fragments [13].

The notion of a differential regulation of progranulin levels in CSF and serum is moreover supported by our observation that the progranulin-albumin ratio (defined by the quotient of progranulin concentration and albumin concentration) was approximately four times higher in CSF than in serum (Table **1**), both in patients and in controls (Appendix **B**). The higher progranulin-albumin ratio in CSF compared to serum also indicates that the CSF progranulin levels are not merely the result of a potential blood contamination, but rather reflect CNS progranulin levels *per se*.

Our findings corroborate and extend recent findings from healthy seniors, which show a differential progranulin regulation between the CNS and the body periphery in healthy ageing [15]. However, we here show for the first time that

Table 2. Characteristics of *GRN*-variant carriers.

Subject	#19869	#13413
phenotype	FTD	FTD
chromosomal position	exon 10, chr17:42428972_42428973	exon 7, chr17:42428147
cDNA change	c.988_989dupAC	c.687T>G
protein change	p.Q331RfsX31	p.Y229X
GVS function	frameshift insertion, leading to premature stop	stopgain
MAF EVS 6500	n. a.	n. a.
ExAC	n. a.	n. a.
PhyloP	n. a.	non-deleterious (0.12)
LRT	n. a.	neutral (0.87)
Mutationtaster	disease causing (1)	disease causing (1)
Polyphen-2	n. a.	possibly damaging (0.57)
SIFT	n. a.	0 (deleterious)
transcript	NM_002087, CCDS11483	NM_002087, CCDS11483
serum progranulin	16.9 ng/ml	1.5 ng/ml
CSF progranulin	2.0 ng/ml	1.8 ng/ml

In two FTD patients, likely pathogenic variants of *GRN* were identified by Sanger sequencing. We used the algorithms SIFT, PhyloP, Polyphen-2, Mutationtaster, LRT to predict the pathogenicity of the variants (for details, see Appendix A: *GRN* sequencing/exome analyses).

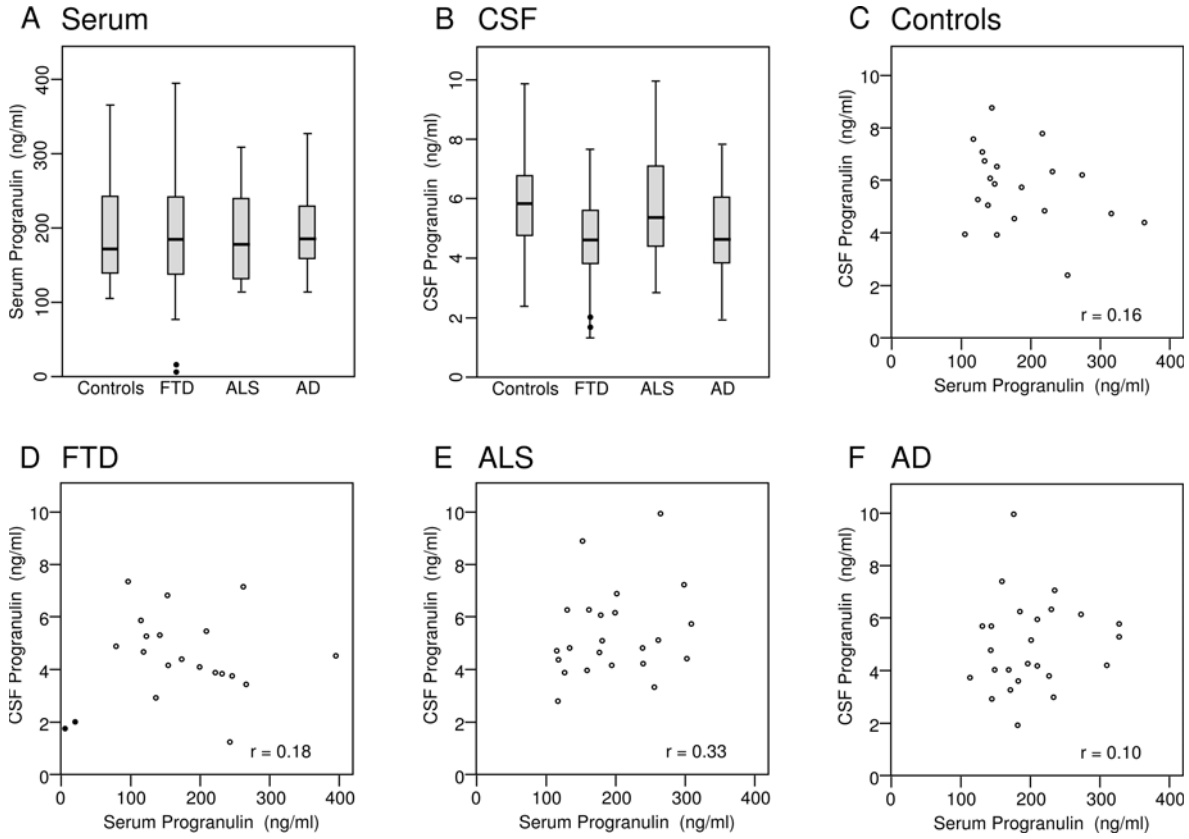


Fig. (1). Serum and cerebrospinal fluid levels of progranulin. The boxplots illustrate the progranulin levels (ng/ml) in serum (A) and cerebrospinal fluid (B) in frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Alzheimer's dementia (AD) and controls. Central horizontal lines hereby indicate median values. Boxes illustrate the ranges between lower and upper quartiles. Error bars represent the full ranges of data without outliers. Filled black circles indicate FTD subjects carrying likely pathogenic *GRN* variants (A, B, D). The scatterplots depict the relation between serum and cerebrospinal fluid progranulin levels (r: Pearson partial correlation coefficient, corrected for age and gender) within the groups of controls (C), FTD patients (D), ALS patients (E) and AD patients (F).

this differential progranulin regulation between the CNS and the body periphery is true also for several neurodegenerative diseases previously linked with progranulin alterations. Here, a thorough and specific understanding of progranulin regulation is of particular importance for uncovering its pathogenic role in neurodegenerative processes and for developing future treatment strategies. The exact mechanisms underlying this differential progranulin regulation remain to be ascertained as the presence of neurodegeneration *per se* did not sufficiently explain the variability between serum and CSF progranulin levels. The lack of correlation might, at least in part, be explained by the fact that progranulin levels in peripheral tissues are subject to other factors than progranulin levels in CSF, such as systemic proliferation, metabolism and inflammation [1, 18]. Moreover, also intrathecal progranulin production and secretion may differ between individuals, depending on the neurodegenerative disease and the individual's disease stage, which could contribute to the missing correlation between CNS and peripheral progranulin levels. However, our study has to leave the exact mechanisms open to future studies investigating genetic and non-genetic contributing factors in larger cohorts.

Nevertheless, our study already yields important implications for clinical practice and research. The observed differential regulation of progranulin in CSF compared to serum in all three neurodegenerative diseases implies that changes of serum progranulin may not adequately capture changes of the progranulin processes in patients' CSF and, probably, in their CNS tissue [15]. Thus, while plasma progranulin certainly is a well-established and extremely helpful biomarker for detecting *GRN* LoF mutations [13, 14, 19], our results advise caution when making inferences from serum to CNS progranulin levels and to its pathogenic role in the large field of neurodegenerative diseases which are not caused by LoF *GRN* mutations. Likewise caution is necessary when making inferences from CSF to CNS *parenchymal* progranulin levels. Although CSF directly surrounds the brain tissue and – unlike peripheral blood – is less subject to systemic influences, such as peripheral systemic metabolism and inflammation [2, 20], CSF progranulin levels still may not necessarily accurately represent the progranulin levels within the CNS tissue. While CSF surrounds the *whole* CNS, brain tissue progranulin concentrations even vary between different brain *regions*, particularly in neurodegenerative disease [21, 22]. In fact, progranulin concentrations may even vary between CNS cell types, particularly between neurons and microglia [23, 24]. Unfortunately, no brain tissue was available from the subjects of our cohort to determine the progranulin levels in different brain regions and brain cell types and to compare them to the respective CSF and serum values. Thus,

it is possible that neither CSF nor serum values of progranulin closely correlate with its genuine values in different brain areas and CNS cell types.

The missing close association between central and peripheral progranulin also needs to be considered with regard to future treatment trials. It has been proposed that enhancing progranulin levels in FTD and AD could serve as a novel treatment strategy [2, 6, 8]. For example, drugs like chloroquine, nimodipine and vorinostat have been demonstrated to increase progranulin levels [25, 26]. While this is certainly promising, our results demonstrate that *blood* levels of progranulin and their increase might not serve as a valid marker for these therapies as they do not necessarily allow to reliably predict the CNS response to progranulin-increasing therapeutics (at least not in non-*GRN*-mutation-carriers); this response, however, would be essential for treating neurodegenerative disease.

APPENDIX A: SUPPLEMENTAL METHODS

Ethics Statement

The university's ethics committee approved the study and all subjects gave written informed consent.

Serum and CSF Samples

CSF and serum samples were obtained from the local Neuro-Biobank and analysed according to established procedures [27]. CSF and blood samples were taken within a time interval of max. 20 minutes. Specifically, CSF was collected by lumbar puncture between 08:00 am and 10:00 am, centrifuged and stored at -80°C within 60 min after collection. Two CSF samples with increased cell count (> 10/µl) were excluded from the final CSF analysis.

Progranulin ELISA

To determine serum and CSF progranulin levels, we used a human progranulin ELISA kit according to the manufacturer's protocol (Adipogen AG, Liestal, Switzerland). We analysed all samples in duplicate and used the provided recombinant human progranulin as a standard. CSF samples were diluted 1:10 and serum samples were diluted 1:200 in the dilution buffer provided by the manufacturer. Accordingly, we multiplied the primary measurements by the respective dilution factor. The ELISA had the following characteristics: progranulin detection limit 32 pg/ml, assay range 0.063 – 4 ng/ml, intra-assay coefficient of variation < 6.93 %, inter-assay coefficient of variation < 7.32 % (source: manufacturer, Adipogen AG, Liestal, Switzerland). Therefore, measurements were possible in the following ranges: 0.63 - 40.0 ng/ml for CSF and 12.6 - 800 ng/ml for serum. All CSF progranulin levels were within the range for CSF measurements. All but one serum progranulin levels were within the range for serum measurements, with the single exception being subject #13413, whose serum level (1.5 ng/ml) was below the lower limit of the range for serum measurements. The polyclonal ELISA antibody was validated elsewhere [20], has been successfully used for plasma measurements previously [13, 14] and does not detect granulins or other progranulin fragments [13].

Progranulin Westernblot

Serum samples were diluted 1:10 in ultrapure water and depleted from albumin according to the manufacturer's instructions (Pierce Albumin Depletion Kit, Thermo Fisher Scientific, following Finch *et al.* 2009, Brain) because albumin with its molecular weight of 66 kDa would have located close to progranulin and, given its high serum concentration, would have prohibited the reliable detection of progranulin in the Westernblot. Only the fraction from the first depletion step was used for the Westernblot analysis, as it contained the highest progranulin levels (data not shown). Undepleted CSF samples and albumin-depleted serum samples were diluted 1:2 in NuPAGE 2x LDS sample buffer containing 10% 2-mercaptoethanol and loaded on 8% Bis-Tris gel (Bolt Bis-Tris Plus, Thermo Fisher Scientific). We used the recombinant human progranulin (0.4 ng) provided with the ELISA kit as a standard (AG-45A-0018PP-KI01, Adipogen, Switzerland). The gels were run with MOPS SDS buffer and subsequently transferred onto nitrocellulose membranes. The blots were blocked in PBS containing 0.05% Tween and 4% (w/v) skim milk powder for 45 minutes at room temperature and probed with the polyclonal ELISA detection antibody against human progranulin (AG 101, Adipogen, referred to as primary antibody) 1:1000 in PBS-T at 4°C overnight. The following day, the membranes were incubated in the secondary antibody solution (peroxidase-conjugated AffiniPure donkey anti-rabbit IgG, Jackson ImmunoResearch Laboratories) 1:20000 in PBS-T. The proteins were then detected with ECL prime (GE Healthcare Life Sciences) and visualised on films (Kodak Biomax XAR films, Carestream Health, see Appendix **D**). Serum progranulin bands were quantified on scans of the films using ImageJ (version 1.50c, http://imagej.nih.gov/ij/).

GRN Sequencing / Exome Analyses

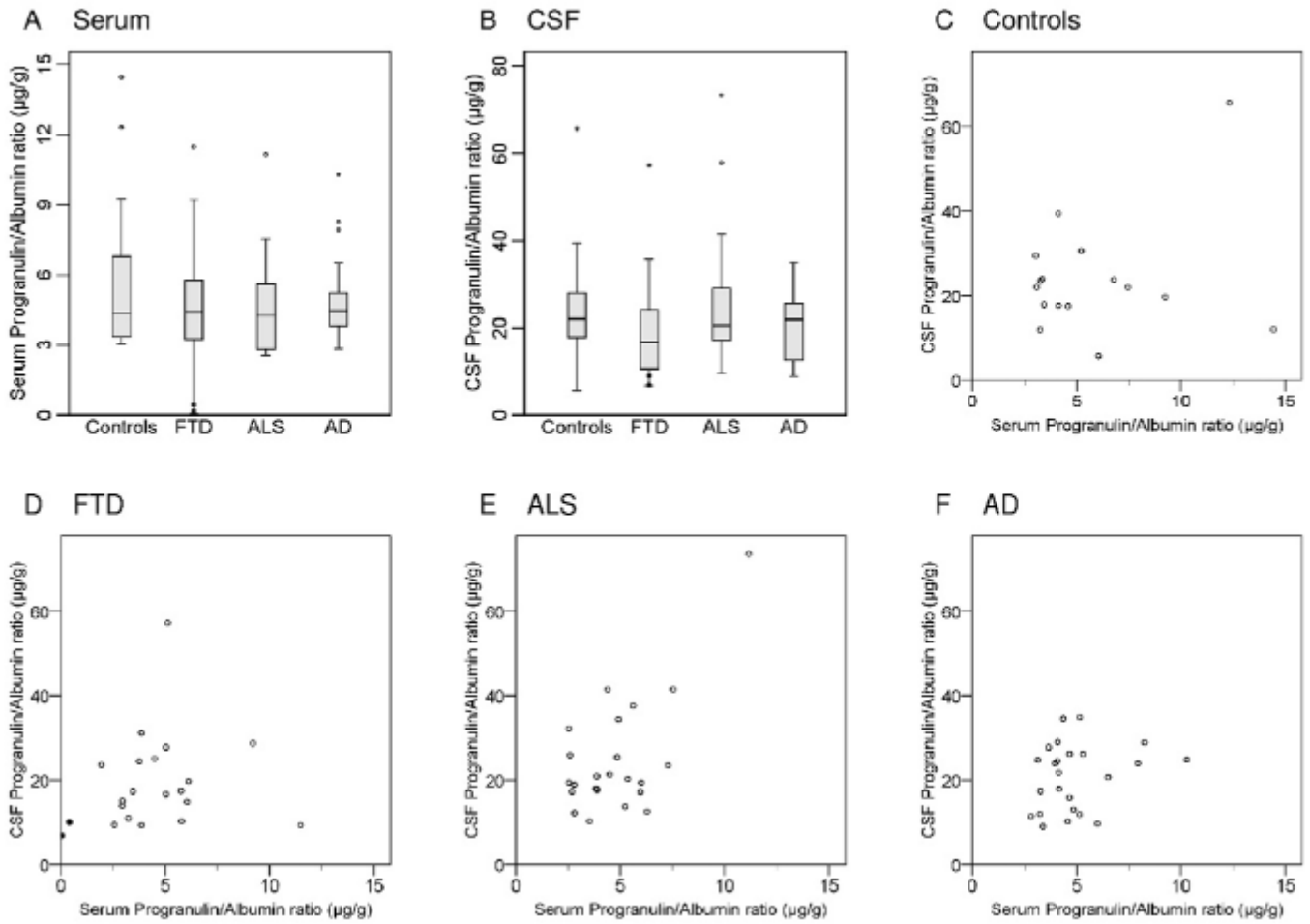
We screened for *GRN* mutations in 3 subjects (2 FTD patients, 1 control) by direct sequencing of *GRN*. The entire coding region and the flanking exon-intron boundaries of *GRN* were amplified by PCR method and screened by direct bidirectional sequencing according to standard protocols using the 3730xl DNA Analyzer (Applied Biosystems). Primer sequences are noted below.

In 2 FTD patients, *GRN* was screened as part of whole exome sequencing. The SureSelect Human All Exon version 5 (Agilent) was used for in-solution enrichment and exome sequencing was performed by using the HiSeq2000 instrument (Illumina), producing 100-bp length paired-end reads. BWA and GATK software packages [28-30] were used to align sequence reads to the reference (UCSC hg19) and call variant positions, respectively. Mean coverage of *GRN* was > 100x. The called variants were annotated with ANNOVAR [31]. Only variants within exons or at splice-sites were considered. Furthermore, *GRN* variants were excluded when being synonymous or having a minor allele frequency above 0.1 % in public databases (1000G [32], EVS [33], ExAC [34]). The algorithms SIFT [35], PhyloP [36], Polyphen-2, Mutationtaster [37], and LRT [38] predicted the pathogenicity of the variants. *GRN* variants of interest were confirmed by Sanger sequencing.

Primer	Sequence
PGRN-E01F	GGCGCCTGCAGGATGGGTTA
PGRN-E01R	CGGTTCTTCTGTTGTCTCCGGCTGA
PGRN-E02F	CAGGGAGGTGTTGAGAAGGCTCAGG
PGRN-E02R	TGGGCCATTTGTCCTAGAAAGACAGG
PGRN-E03F	GAGCTGGCAGCCTGGGTTTTCC
PGRN-E03R	CCTGTGGAACACAGAGAAACCTGCATT
PGRN-E04F	CCACTGCTCTGCCGGCCACT
PGRN-E04R	CCCCAGTGCTGCCCCTCTGT
PGRN-E05+06F	GCTGAGGGAGGGACTGGATTGTGA
PGRN-E05+06R	GGCCACTGGAAGAGGAGCAAACG
PGRN-E07+08F	TGAGGAGGTGGGAGAGCATCAGG
PGRN-E07+08R	TCAACCCTTTGCCGGCTCCA
PGRN-E09+10F	CCAGCTGTGGAGCCGGCAAA
PGRN-E09+10R	TGCCGAGCCCCTACCTACTCCA
PGRN-E11F	GTCCCCAGCTGGAGGTGCTGTAAG
PGRN-E11R	TGGCATTATGTTCCTGTCCCCTCAC
PGRN-E12F	CTCCCTGCCTGCCCCTGGAT
PGRN-E12R	GGGGCGAGAGGGTTGGACGA
PGRN-E13F	CCCCATCCTGGGGCTGGGTA
PGRN-E13R	TGAAACGCACACGCGCACAC
PGRN-E05+06FS1	TCCCTGAGTGGGCTGGTAGTATCCTG
PGRN-E13FS1	TGGGTATGGCCAGGGACCAG
PGRN-E05RS	AACTCTGCCCCCACTTCCCTC

Statistical Analyses

We statistically analysed the data with SPSS (IBM, Version 22). Group effects on serum progranulin and CSF progranulin, respectively, were tested with two separate independent one-way analyses of variance (ANOVAs) and, if applicable, post-hoc t-tests (Bonferroni-corrected for multiple comparisons). In the subset of subjects for whom both serum and CSF were available, we summarised the association between serum progranulin and CSF progranulin by Pearson partial correlation coefficients (partial r), controlling for subjects' age and gender [15, 16]. We analysed the progranulin-albumin ratio in both serum and CSF with non-



parametric tests, using Kruskal-Wallis test for group effects and Spearman's rank correlation for the association between serum and CSF ratios within groups.

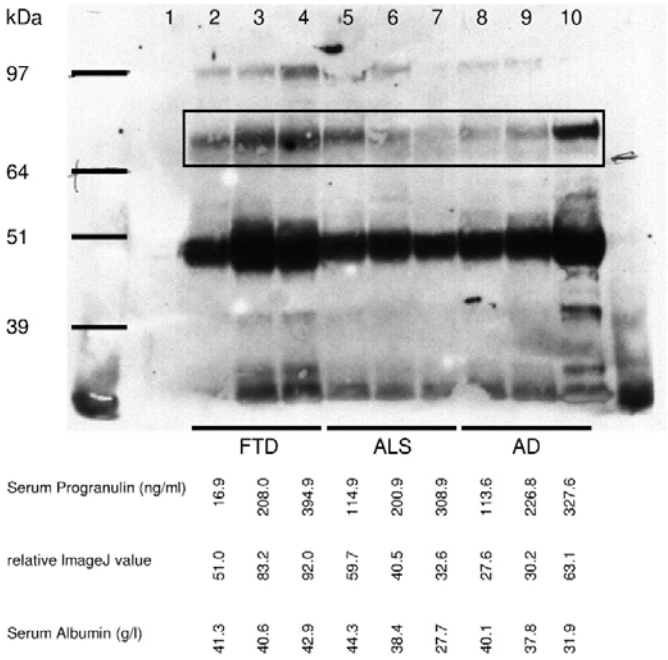
APPENDIX B: SUPPLEMENTAL RESULTS

Serum progranulin levels did not differ significantly between groups (independent one-way ANOVA, $F(3, 96) = 0.689$, $p = .561$; Fig. **1A**, Table **1**). Likewise, the serum progranulin-albumin ratio did not differ significantly between groups (Kruskal-Wallis-test, $\chi^2(3) = 1.363$, $p = .714$; Table **1**). While a significant group effect was observed for CSF progranulin levels (independent one-way ANOVA, $F(3, 118) = 3.349$, $p = .021$; Fig. **1B**, Table **1**), post-hoc t-tests did not reveal any significant differences between groups (all $p > .05$, Bonferroni-corrected for multiple comparisons). The CSF progranulin-albumin ratio also did not differ significantly between groups (Kruskal-Wallis-test, $\chi^2(3) = 6.558$, $p = .087$; Table **1**).

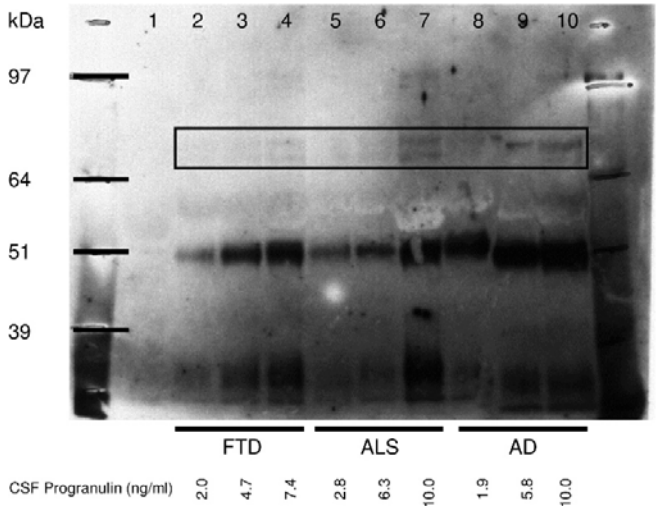
APPENDIX C: PROGRANULIN-ALBUMIN RATIOS IN SERUM AND CEREBROSPINAL FLUID

The boxplots illustrate the progranulin-albumin ratios (μg/g) in serum (A) and cerebrospinal fluid (CSF) (B) in frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Alzheimer's dementia (AD) and controls. Central horizontal lines hereby indicate median values. Boxes illustrate the ranges between lower and upper quartiles. Error bars represent the full ranges of data without outliers. Filled black circles indicate FTD subjects carrying likely pathogenic GRN variants (A, B, D). The scatterplots depict the relation between serum and cerebrospinal progranulin-albumin ratios within the groups of controls (C), FTD patients (D), ALS patients (E) and AD patients (F).

APPENDIX D: PROGRANULIN WESTERNBLOT



(1) Detection of various progranulin concentrations in serum. Westernblotting of albumin-depleted serum samples with the polyclonal ELISA detection antibody against recombinant human progranulin (AG 101, Adipogen) demonstrated the expected bands for progranulin (box), located above 64 kDa (for methods, see Appendix **A**). The progranulin band was consistently observed in the serum samples of nine exemplary patients (three from each neurodegenerative disease group) with various individual progranulin serum concentrations (as quantified by our initial ELISA, concentrations reported below Westernblot, columns 2-4: FTD, 5-7: ALS, 8-10: AD). Hereby increasing progranulin concentration should correlate with increasing thickness and density of the progranulin bands. This association was observed in the three FTD samples and the three AD samples, but not in the three ALS samples. We confirmed the visually observed association by quantification of the progranulin bands with ImageJ (results reported below Westernblot). Band size of the progranulin band could not be explained by albumin (which would also locate around 64 kDa and which might have partially remained despite prior albumin depletion), as progranulin band size was not associated with albumin concentration in the undepleted serum samples (albumin concentrations in undepleted serum reported below Westernblot). Additional bands were observed, particularly bands around 51 kDa, which likely corresponded to IgG heavy chain as an abundant serum protein. When we omitted the ELISA antibody from the Westernblot procedure (all other parameters being constant), these additional bands were reproduced, while the progranulin band above 64 kDa was lost (data not shown), suggesting unspecific binding of the secondary Westernblot antibody (peroxidase-conjugated donkey anti-rabbit IgG) to various serum proteins, but specific binding of the ELISA detection antibody to serum progranulin. The loaded quantity of recombinant human progranulin (0.4 ng, column 1) failed to reach the detection limit of the Westernblot. In summary, the Westernblot findings for serum were compatible with our initial ELISA measurements, although the Westernblot method was inherently less sensitive and less quantitative than the ELISA method.



(2) Detection of various progranulin concentrations in CSF. Westernblotting also showed the progranulin band (box) in the CSF samples of nine exemplary patients (three

from each neurodegenerative disease group). The corresponding individual progranulin CSF concentrations yielded by our initial ELISA are reported below the Westernblot (columns 2-4: FTD, 5-7: ALS, 8-10: AD). As expected, CSF progranulin concentrations were close to the detection limit of the Westernblot. Nevertheless, increasing progranulin CSF concentration was associated with increasing thickness and density of the progranulin band in all three patient groups. Thus, the Westernblot results are in line with our ELISA measurements. Given the heterogeneous background and given the lack of linearity between band size and concentration for concentrations close to the detection limit, progranulin bands were not quantified with Image J.

AUTHOR CONTRIBUTIONS

Dr. Wilke: design and conceptualisation of the study, acquisition of data, analysis of data, drafting of manuscript.

Dr. Gillardon, Mr. Deuschle, Mrs. Dubois, Mr. Hobert, Dr. Müller vom Hagen, Mrs. Krüger, Dr. Biskup, Mr. Blauwendraat, Mr. Hruscha, Dr. Heutink, Mr. Kaeser and Dr. Maetzler: acquisition of data, analysis and interpretation of data, revision of manuscript.

Dr. Synofzik: design and conceptualisation of the study, acquisition of data; analysis and interpretation of data, revision of manuscript.

CONFLICT OF INTEREST

Dr. Gillardon is employee of Boehringer Ingelheim Pharma GmbH & Co KG, CNS Diseases Research, Biberach an der Riss, Germany. This company has no direct market-related interests in this study. Dr. Müller vom Hagen received speaker's honoraria from Actelion Pharmaceuticals Ltd. Dr. Biskup is founder and managing director of CeGaT GmbH, Center for Genomics and Transcriptomics, Tübingen, Germany. This company has no direct market-related interests in this study. Stefanie Krüger is employee of CeGaT GmbH. Dr. Maetzler received speaker's honoraria from UCB and GSK and funding from the European Union, the German Federal Ministry of Education and Research, the Robert Bosch Foundation, Janssen Pharmaceutica and the Michael J. Fox Foundation. Dr. Synofzik received consulting fees from Actelion Pharmaceuticals Ltd. All other authors do not report financial disclosures.

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Early-onset parkinsonism due to compound heterozygous *POLG* mutations

Keywords:
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Compound heterozygosity for two specific mutations (*G737R*, *R853W*) in the nuclear polymerase gamma-1 (*POLG*) - gene, which encodes for the catalytic subunit of the mtDNA polymerase gamma [1], has been associated with a syndrome consisting of early-onset parkinsonism, anxiety and axonal polyneuropathy as well as ragged red fibers and COX-deficient fibers in muscle biopsy in two sisters [2]. In contrast to all other pathologic *POLG* mutations [1], these patients did not show signs of chronic progressive external ophthalmoplegia (CPEO) [2]. We present the first replication of this genotype-phenotype association:

A 32-year-old woman presented with a syndrome consisting of parkinsonism (slightly stooped posture, facial masking, moderate brady- and -dysdiadochokinesia pronounced on the right side as well as slight right-sided rigor of the lower extremity) with dystonic toe and plantar flexion. The patient also complained of anxiety and generalized muscle weakness. Symptoms started 5 years earlier and were slowly progressive. The patient gave written informed consent for the scientific use of the presented results. Clinical-neurologic exam did not show signs of paresis but revealed bilateral hypaesthesia of the lateral bottom of the foot and dorsal forefoot as well as bilateral distal pallhypaesthesia of the legs (3/8 at the lateral malleoli).

Double-blind, placebo-controlled levodopa challenge test using 200 mg of levodopa and 50 mg of benserazide [3] showed a dramatic decrease of symptoms in Part III of the Unified Parkinson Disease Rating Scale (UPDRS) [4] with scores of 26 after administration of the placebo in both testings and scores of 0 (1st testing) and 3 (2nd testing) after administration of 200 mg levodopa, respectively (Supplementary Video). The patient also described a decrease of anxiety on a visual analogue scale (0 = no anxiety; 10 = maximum anxiety; 8 with placebo vs. 1 with 200 mg levodopa in both testings). Neuropsychological examination on dopaminergic medication showed an impairment of cognitive flexibility and partly of the divided attention with borderline results regarding nonverbal fluency compatible with slight impairment of frontal functions.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2016.04.020.

Ophthalmologic examination showed no clear signs of CPEO with a possible, slight bilateral ptosis (palpebral fissure = 11 mm) and bilateral elevation deficit (25°). The patient also showed poorly compensated exophoria (−1.5° at distance; −6° at near) with decompensation on elevation. These ophthalmologic findings were stable without signs of progression to date (observation period 20 months). There were no signs of retinitis pigmentosa in fundoscopy or electroretinography.

Molecular imaging revealed a striking bilateral absence of nuclide accumulation in both the caudate nucleus and putamen in dopamine transporter scintigraphy with normal postsynaptic nuclide accumulation in IBZM-SPECT and physiological results in MIBG scintigraphy (Supplementary Fig. 1).

Neurography showed signs of slight axonal sensory polyneuropathy. This was confirmed by a biopsy of the sural nerve showing moderate clearing of fiber concentration with even loss of thick and thin fibers corresponding to moderate predominantly axonal chronic neuropathy.

Muscle biopsy of vastus lateralis showed mild variation in fiber diameter, a few scattered ragged-red fibers, slight accumulation of lipid droplets, numerous COX-deficient fibers and some lobulated fibers. On electron microscopy, aggregates of partly atypical mitochondria were found, some of which demonstrated electron dense inclusions, whereas others showed blurred cristae and sharply demarcated electron dense outer membranes. Fat droplets were frequently present in close vicinity to the organelles. Autophagic vacuoles were noted in some of the muscle fibers (Supplementary Fig. 2).

Panel sequencing of gene loci associated with hereditary parkinsonism [5] showed the patient compound heterozygous for two pathogenic mutations (*c.2209G >C*; *p.G737R* (het.) in exon 13 and *c.2557C >T*; *p.R853W* (het.) in exon 16) in the *POLG* − gene (GenBank accession number NM_002693.1), as described earlier [2], with segregation analysis revealing paternal heterozygosity for *G737R* and maternal heterozygosity for *R853W*, respectively.

In conclusion, this case report replicates the reported association between the described genotype (compound heterozygosity for the *POLG* mutations *G737R* and *R853W*) and its corresponding phenotype (early-onset parkinsonism with dystonic toe curling and concurrent anxiety, axonal neuropathy as well as ragged-red fibers and COX-deficient fibers in muscle biopsy) [2] for the first time (Table 1). Just like previously reported, our patient also did not show clear signs of CPEO, which is surprising, as CPEO is a key feature in other *POLG*-mutations [2]. With the close reproduction of previously described features, further proof is given that compound heterozygosity for *POLG* mutations *G737R* and *R853W* causes a phenotype characterized by levodopa-responsive early-onset parkinsonism. Therefore, we propose that the disease

http://dx.doi.org/10.1016/j.parkreldis.2016.04.020

Table 1
Symptoms and findings of the three patients compound heterozygous for *POLG* mutations *G737R* and *R853W*.

	Davidzon et al. [2]		Rempe et al.
	Patient 1	Patient 2	Patient 3
Beginning of symptoms	26	20	27
Symptoms of parkinsonism	Stooping, shuffling gait, bradykinesia, positive pull test, facial masking, hypophonia, cogwheeling of wrists	Stooped posture, bradykinesia, rigidity, facial masking	Stooped posture, brady- and dysdiadochokinesia, rigor of the lower extremity, facial masking
Tremor	Postural action tremor of all limbs	Intermittent action tremor of all limbs	No tremor
dystonic symptoms	Dystonic toe curling	Dystonic toe curling	Dystonic toe curling and plantar flexion
Psychological symptoms	Anxiety	Anxiety, depression	Anxiety
Levodopa responsiveness	Responsive to pramipexole (levodopa not tried)	Levodopa-responsive with higher doses causing oral dyskinesia	Levodopa-responsive
Peripheral polyneuropathy	Axonal predominantly sensory neuropathy	Axonal sensorimotor, predominantly sensory neuropathy	Axonal sensory neuropathy
Findings in muscle biopsy	2 to 3% RRF Cytochrome c oxidase deficient fibers Focal fiber–type grouping and scarce targetoid structures. Decreased activities of respiratory chain complexes containing mtDNA-encoded subunits decreased (varying from 10 to 18%)	1 to 2% RRF Cytochrome c oxidase deficient fibers Mild focal fiber–type grouping, and sparse targetoid fibers. Decreased activities of respiratory chain complexes containing mtDNA-encoded subunits decreased (varying from 40 to 60%)	Few scattered RRF Numerous cytochrome c oxidase deficient fibers Mild variation in fiber diameter, some lobulated fibers, autophagic vacuoles in some of the muscle fibers. Aggregates of atypical mitochondria with electron dense inclusions/outer membranes, blurred cristae and fat droplets in close vicinity

RRF = ragged red fibers.

caused by *G737R* and *R853W* should be listed among the monogenic parkinsonisms and should be included in the *PARK* – nomenclature.

Authors' roles

T. Rempe, G. Deuschl and T. van Eimeren treated the patient. S. Krüger and S. Biskup executed the genetic analyses. J. Matschke and C. Hagel performed the histological analyses. C. Hagel drafted the histological figure. T. Rempe and T. van Eimeren drafted the manuscript. All authors critiqued and revised the manuscript and have agreed with the final version of the paper.

Financial disclosure/Conflict of interest

No specific funding was received for this work. The authors declare that there are no conflicts of interest relevant to this work. Unrelated to this research, G. Deuschl has received honoraria from Medtronic, Desitin and UCB and has been serving as a consultant for Medtronic and Boston Scientific. He received royalties from Thieme Publishers. He receives through his institution funding for his research from the German Research Council, the German Ministery of Education and Health and Medtronic. T. van Eimeren has received honoraria from Lilly Pharmaceuticals and has been serving as a consultant for the CHDI Foundation and Lilly Pharmaceuticals. He receives research support from the German Research Council. G. Kuhlenbäumer reports funding by DFG (KU 1194/9-1, KU 1194/5-1) and by intrauniversity grants of the Christian-Albrechts-University Kiel. T. Rempe, S. Biskup, S. Krüger, J. Matschke and C. Hagel have nothing to disclose. All authors are government employees except for S. Biskup and S. Krüger who are employees of the Center for Genomics and Transcriptomics, Paul-Ehrlich-Strasse 23, 72076 Tübingen, Germany.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2016.04.020.

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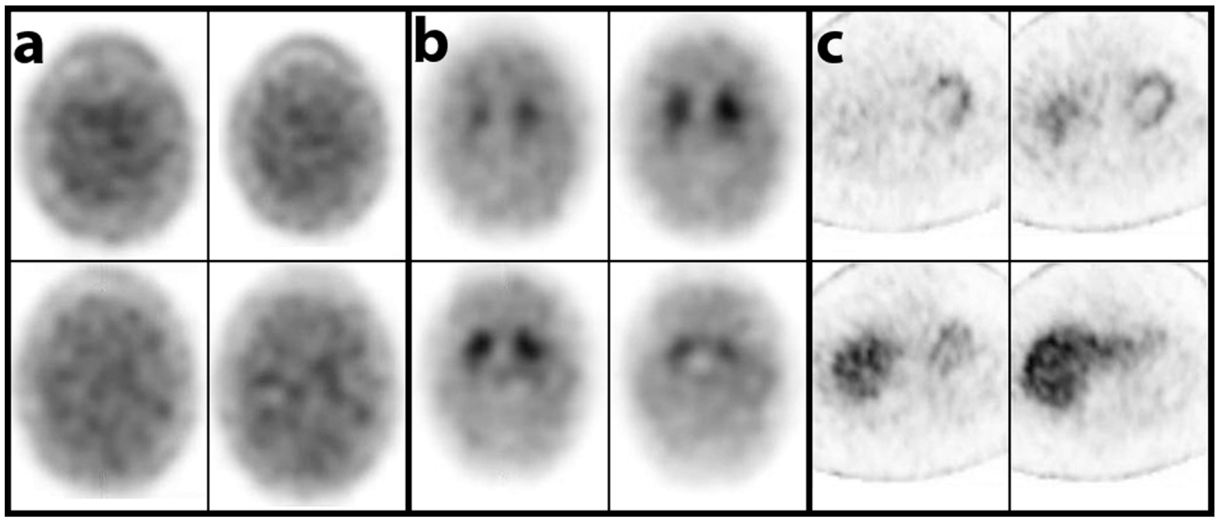
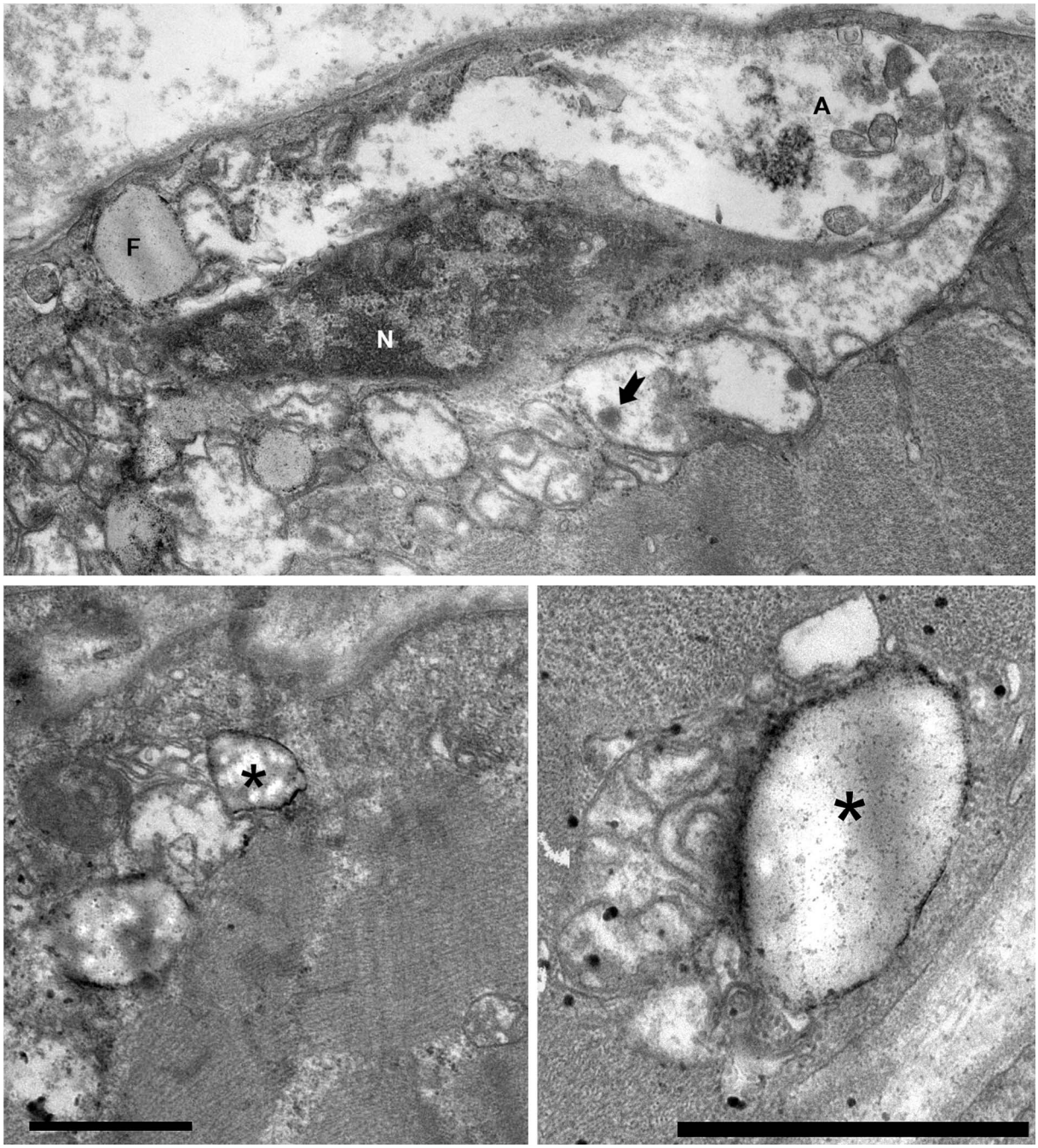
Appendix A. Supplementary data

The following are the supplementary data related to this article:



Supplementary Fig. 1.

Dopamine transporter scintigraphy (a), IBZM - (b) and MIBG - (c) SPECT. Dopamine transporter scintigraphy shows bilateral absence of nuclide accumulation in both the caudate nucleus and putamen, IBZM- and MIBG – SPECT show physiological results. SPECT = Single-photon emission computed tomography; IBZM = iodobenzamine; MIBG = metaiodobenzylguanidine.



Supplementary Fig. 2.

Electron microscopy of muscle biopsy. Upper micrograph: Subsarcolemmal autophagic vacuole (A) near nucleus (N) with neighboring fat droplet (F) and a large cluster of mitochondria showing rarified cristae and infrequent electron dense inclusions (arrow); lower left and right micrographs: Mitochondria with sharply demarcated electron dense outer membrane and blurred cristae (asterisks). Scale bars: 1 µm, lower left scale bar applies also to upper micrograph.

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Novel cases of amyotrophic lateral sclerosis after treatment of cerebral arteriovenous malformations

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Summary

Previous case studies reported nine patients with cerebral arteriovenous malformations (AVM) who developed amyotrophic lateral sclerosis (ALS) after AVM embolisation. Here, we describe three novel cases of ALS which developed 13–34 years after treatment, including embolisation, of cerebral AVM. This study provides further arguments supporting the thesis that embolisation of cerebral AVM might influence the risk of later ALS development.

Key words: *arteriovenous malformation; amyotrophic lateral sclerosis; ALS; embolisation*

Background

We reported previously that seven patients with cerebral arteriovenous malformations (AVM) for whom embolisation was utilised as the form of treatment developed amyotrophic lateral sclerosis (ALS) after a median latency of 14 years [1]. The patients were unusually young at ALS onset (median age 38 years) and had an AVM with perinidal angiogenesis and multiple embolisation sessions. We speculated that a reduction of the synthesis of vascular endothelial growth factor (VEGF) after AVM embolisation [2] may have been the underlying mechanism. Meanwhile, Katsavarou and colleagues reported two other young patients who developed ALS 11 and 14 years after their first cerebral AVM embolisation [1]. Here, we describe three novel cases of ALS which developed 13–34 years after treatment of cerebral AVM.

Cases

Patient 1

A 34-year-old woman developed a secondarily generalised epileptic seizure and was diagnosed with an AVM with perinidal angiogenesis in the left middle frontal gyrus. Two partial embolisations were performed: one shortly thereafter and another 19 years later (fig. 1a, b). At the age of 54, she developed bulbar onset ALS meeting the criteria for definitive ALS [3]. No ALS or AVM was reported in her family history. Sanger sequencing of the entire coding region and flanking intronic regions of the *VEGFA* gene (ENST00000372055, CCDS34457) in accordance with standard protocols revealed no variation.

Patient 2

A 37-year-old woman experienced headaches resulting in the diagnosis of an AVM of the precentral gyrus. The AVM was embolised in four sessions before being surgically removed (fig. 1c). Thirteen years later, at the age of 50, she

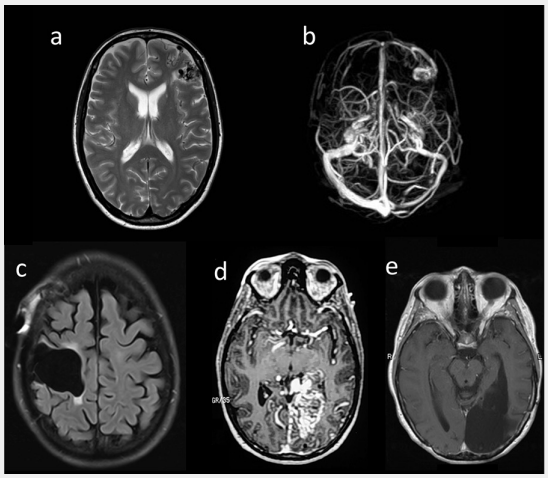

Figure 1

(a, b) Patient 1: T1-MRI (magnetic resonance imaging) (a) and MRI-venogram (b); (c) Patient 2: MRI (FLAIR) after surgery; (d, e) Patient 3: MRI-angiography prior to surgery (d) and MRI (T1) after surgery (e).

developed ALS starting in her left arm, with fatal outcome after 3 years. Post-mortem examination of the central nervous system confirmed classical ALS pathology with neuronal and glial TAR-DNA binding protein (TDP)-43 inclusions predominantly in the upper and lower motor neurons (fig. 2). Notably, no obvious difference in the extent and severity of TDP-43 pathology was seen between the right and left precentral gyrus (fig. 2A, B). No immunoreactive inclusions were detected with antibodies against dipeptide repeat proteins (poly-GA), the highly characteristic feature of C9orf72 repeat expansion carriers or with antibodies against FUS (an RNA binding protein), thereby excluding relevant *C9orf72* and *FUS* gene mutations. There was no mutation of the *VEGFA* gene.

Patient 3

A woman with headaches and a visual field deficit was found to have a left-sided medial occipital AVM, and at the ages 20 and 21 years, respectively, two embolisations with silastic spheres were done. She returned at the age of 36 with severe headaches and complete right homonymous hemianopia (fig. 1d). She then underwent three embolisation sessions over a 1-week period with a combination of cyanoacrylate, polyvinyl alcohol particles and platinum microcoils followed by complete surgical resection. At age 53, she began to develop right arm twitching, atrophy and weakness. A diagnosis of definite ALS was made. The patient refused genetic analyses.

Conclusion

We extend the existing descriptions of an association between cerebral AVM embolisation and deferred ALS with an onset ranging up to 34 years after the procedure and without correlation between the side of ALS limb onset and the location of the AVM in the brain. Therefore, the connection between AVMs and ALS cannot be explained solely by local, but rather by systemic factors. Interestingly, no ALS has so far been reported in AVM patients treated with surgery or radiotherapy alone or with complete embolisation. AVMs lead to increased local angiogenic activity associated with increased VEGF expression. This was apparent in the perinidal angiogenesis in all seven cases of AVM and ALS in the initial study [1]. Our results extend these numbers by two further cases (no information for patient 3). We conclude that embolisation of cerebral AVMs with perinidal angioneogenesis might induce mechanisms such as lowering of the VEGF level [2] and thereby might influence the risk of ALS development. In addition to AVM or embolisation procedures, cerebrovascular injury from a variety of causes has been suggested to be a risk factor for ALS "within the context of a more complex multiple-hit model of pathogenesis" by Turner et al. [5]. Thus, the mechanism underlying the association of AVM or AVM embolisation and ALS development remains speculative and might depend on specific influences on, for example, VEGF production or on less specific consequences from (vessel-associated?) brain injury.

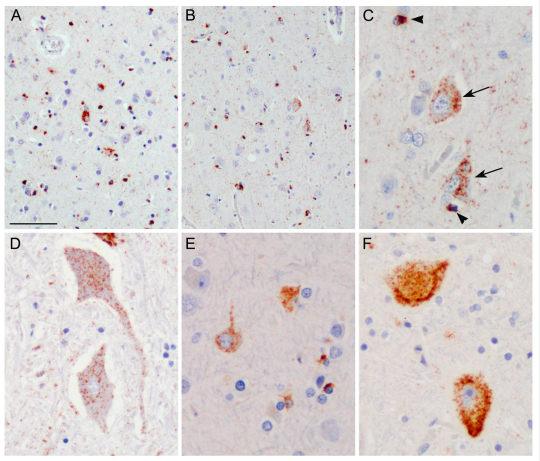

Figure 2

Immunohistochemistry with an antibody against phosphorylated TDP-43.

A–D: Case 2 showing classical amyotrophic lateral sclerosis (ALS) with TDP-43 pathology. A: Left precentral gyrus. B: Right precentral gyrus. C: Higher magnification of B demonstrating TDP-43 positive neuronal cytoplasmic inclusions (arrows) and glial cytoplasmic inclusions (arrowheads). D: Anterior horn of the spinal cord with neuronal cytoplasmic TDP-43 inclusions. E–F: For comparison, TDP-43 staining from the precentral gyrus (E) and spinal cord (F) from an ALS case without an arteriovenous malformation are shown. Scale bar: 80 µm (A, B); 30 µm (C, E), 40 µm (D, F).

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Authors' contribution: M. Linnebank: description of patient 1, draft of manuscript. C. McDougall: description of patient 2. S. Krueger and S. Biskup: genetic analyses. M. Neumann: neuropathology. M. Weller and A. Valavanis: contributed to design of the study and finaliziton of the manuscript. J. Prudlo: description of patient 2, organization of analyses, contribution to finalize the manuscript.

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Figures (large format)

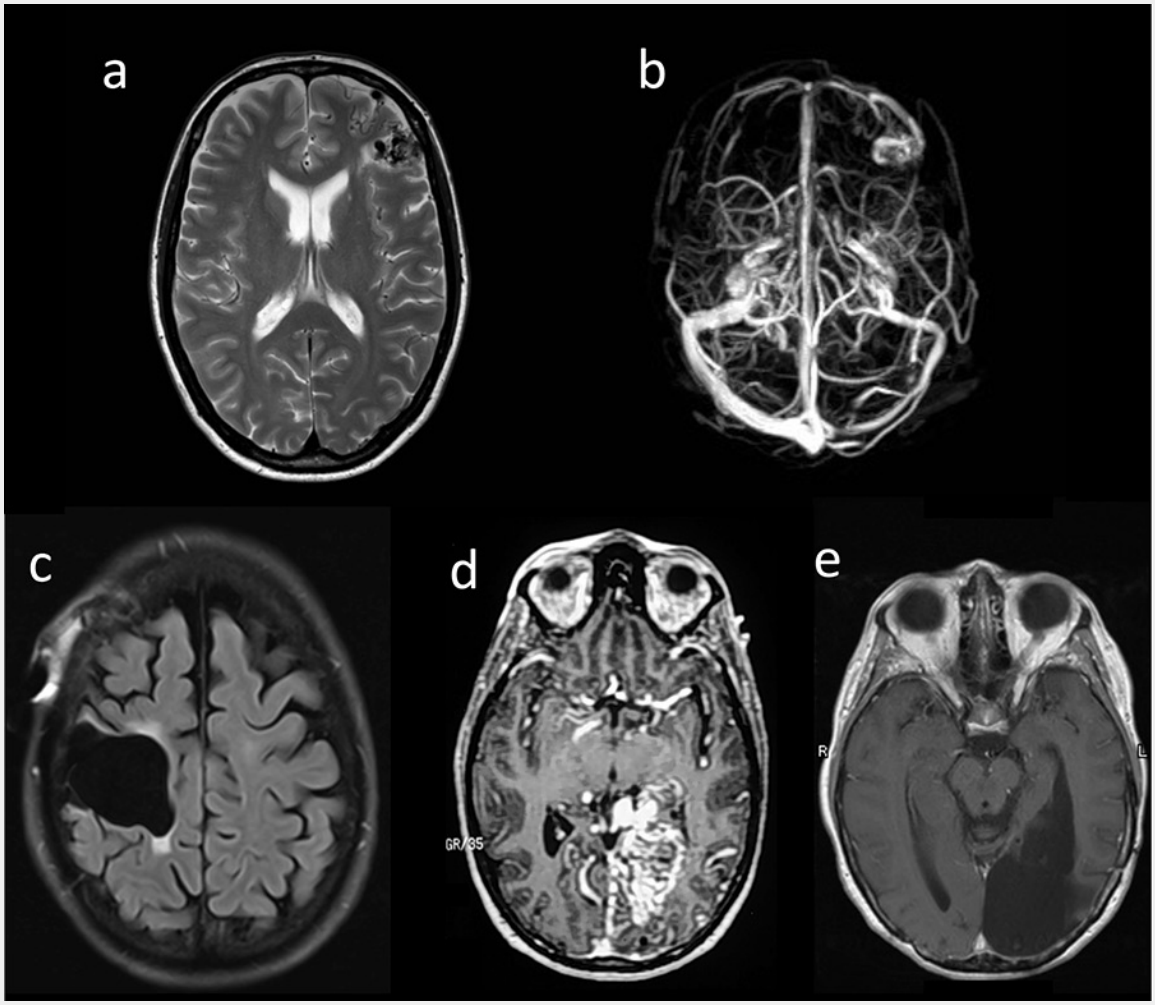

Figure 1

(a, b) Patient 1: T1-MRI (magnetic resonance imaging) (a) and MRI-venogram (b); (c) Patient 2: MRI (FLAIR) after surgery; (d, e) Patient 3: MRI-angiography prior to surgery (d) and MRI (T1) after surgery (e).

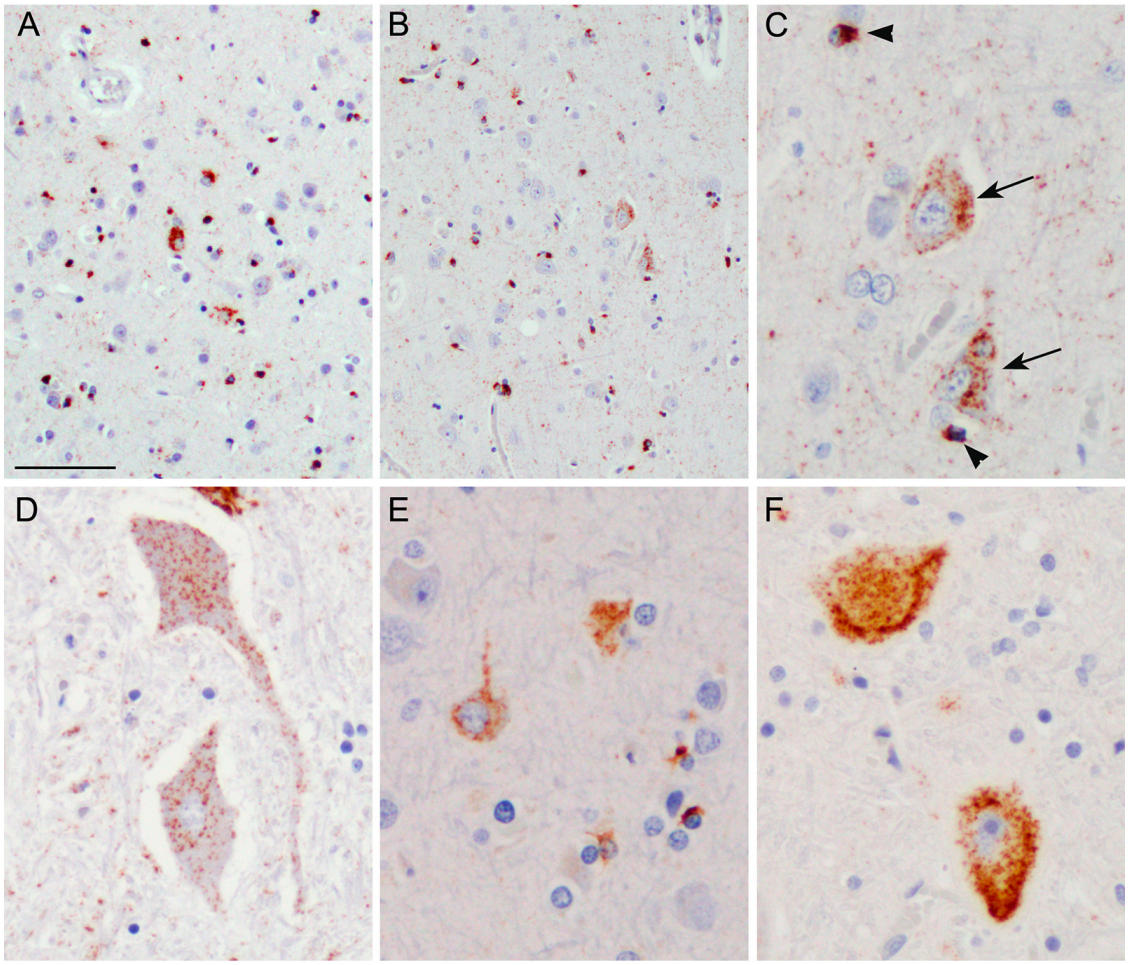

Figure 2

Immunohistochemistry with an antibody against phosphorylated TDP-43.
A–D: Case 2 showing classical amyotrophic lateral sclerosis (ALS) with TDP-43 pathology. A: Left precentral gyrus. B: Right precentral gyrus. C: Higher magnification of B demonstrating TDP-43 positive neuronal cytoplasmic inclusions (arrows) and glial cytoplasmic inclusions (arrowheads). D: Anterior horn of the spinal cord with neuronal cytoplasmic TDP-43 inclusions. E–F: For comparison, TDP-43 staining from the precentral gyrus (E) and spinal cord (F) from an ALS case without an arteriovenous malformation are shown. Scale bar: 80 µm (A, B); 30 µm (C, E), 40 µm (D, F).

Autosomal dominant inheritance in SPG18: Exome sequencing reveals a novel *ERLIN2* missense mutation cosegregating with a pure form of HSP

Running title: Autosomal dominant inheritance in SPG18

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Abstract

Autosomal recessive SPG18 is caused by mutations in the *ERLIN2* gene, encoding the endoplasmic reticulum lipid raft-associated protein 2. To date, SPG18 has been reported only in individuals from few consanguineous families in the Middle East region with complicated forms of autosomal recessive HSP (AR-HSP) or juvenile primary lateral sclerosis (PLS). We report a novel heterozygous missense mutation in *ERLIN2* (c.386G>C, p.S129T) that cosegregates with a pure form of hereditary spastic paraplegia (HSP) and describe for the first time SPG18 with an autosomal dominant inheritance pattern. The mutation was identified in a non-consanguineous German family by a whole-exome sequencing approach. Subsequent candidate mutation validation using Sanger sequencing confirmed the presence or absence of the mutation, respectively, in affected and non-affected family members of three generations. Affected individuals show features of an uncomplicated form of HSP with variable age at onset (range 13 to 46 years) and slow progression. The pathophysiologic mechanism by which the identified mutation causes SPG18 in this family remains elusive. However, the involvement of *erlin-2*, in previous studies localized to ER lipid rafts and linked to ER-associated degradation (ERAD), adds further evidence for specific dysfunctions in the ER network as a common pathogenic mechanism for HSP. The present study expands the mutational, inheritance and phenotypic spectrum of SPG18. We conclude that *ERLIN2* mutations should be considered in the diagnostic evaluation of patients with AD-HSP.

Keywords

hereditary spastic paraplegia (HSP), SPG18, autosomal-dominant, *ERLIN2*, exome sequencing

Introduction

Hereditary spastic paraplegia (HSP) denotes a clinically and genetically highly heterogeneous group of neurodegenerative disorders unified by the defining clinical feature of progressive spasticity and weakness of the lower limbs. Traditionally, HSP is divided into “pure” (uncomplicated) HSP and “complicated” (complex) forms. Pure HSP is characterized by bilateral spasticity in the lower extremities alone, optionally accompanied by mild dorsal column impairment and symptoms of urinary urgency. HSP is classified as complicated if the impairment present in uncomplicated HSP is associated with additional systemic and/or neurological features, including mental retardation, dementia, epilepsy, cerebellar signs, extrapyramidal symptoms, visual dysfunction, deafness, peripheral neuropathy and/or cerebral MRI abnormalities.[1,2] Inheritance of HSP can follow an autosomal dominant (AD), autosomal recessive (AR), X-linked (XL), or even a maternal (mitochondrial) transmission pattern.[2] Most cases of pure HSP are autosomal dominant, whereas complicated forms tend to be autosomal recessive. Autosomal dominant HSP (AD-HSP) is indeed the most common variety, accounting for about 70-80% of all forms of HSP in Western countries, and it is predominantly associated with pure forms. Genetic studies show that HSP is among the most genetically diverse of all diseases, involving at least 75 distinct spastic paraplegia gene loci (SPG) and at least 54 causative genes identified to date. More recently, HSPs are increasingly referred to using a genetic classification (currently designated SPG1-72).[3] Among them, SPG4, 3A, 6, 8, 10, 12, 13, 17, 31, 33, 42, and SPG72 (loci with identified genes) and SPG9, 19, 29, 36, 37, 38, 40, and SPG41 (loci with yet undetermined causative genes) have been associated with autosomal dominant HSP (AD-HSP). Mutations in the genes encoding for SPG4/*spastin*, SPG3A/*atlastin1*, and SPG31/*REEP1* account for the majority of AD forms of HSP, explaining an estimated proportion of up to 50% of AD-HSP.

For the SPG18 locus, *ERLIN2* (ER lipid raft associated 2) has been identified as the causative gene, and mutations have been described associated with a severe form of early-onset complicated AR-HSP [4, 5, 6, 7, 8] and, in one family, with juvenile primary lateral sclerosis (PLS).[9] To date, all *ERLIN2* mutations have been found in individuals from consanguineous families from the Middle East region, and all five causative mutations were detected in a homoallelic state, consistent with an autosomal recessive mode of transmission.

The aim of the present study was to characterize the phenotype and identify the causative gene in a large German family presenting with pure HSP. Using a whole-exome sequencing approach, we identified a novel *ERLIN2* missense mutation in a heterozygous state that cosegregated with a pure and autosomal dominantly transmitted form of HSP in this family. Sanger sequencing confirmed segregation of this mutation with the disease in all

affected family members. Notably, this is the first report of SPG18 with an autosomal dominant inheritance pattern.

Methods

Patients and Probands

Patients and probands were recruited from a large German family, in which individuals with HSP could be pursued over three generations. The pedigree chart is illustrated in Fig. 1. In addition to the index patient IV.7, all available affected (II.1, III.2, III.5, III.6) and non-affected (III.4, IV.3, IV.8) individuals of the extended family were carefully examined by two experienced neurologists (A.D. and F.R.). A detailed medical history was ascertained, including age at onset, presence or absence of clinical features associated with pure or complicated HSP, and progression of disability. Clinical severity and functional impairment were assessed using the Spastic Paraplegia Rating Scale (SPRS; a 13-item scale graded from 0 (normal) to 4 (severe), maximum obtainable score 52)[10] and the Functional Hereditary Spastic Paraplegia Rating Scale (FHSPS)[11, 12], a semi-quantitative instrument to measure disability depending on the landmarks of gait dysfunction (disability stages: 1, no mobility problems or slight stiffness of the legs; 2, moderate gait stiffness; 3, problems running, but able to walk alone; 4, problems in walking; 5, wheelchair-bound).

Two affected family members (III.2, IV.7) underwent extensive work-up including brain and spinal MRI, electroneuromyography (ENG/EMG), EEG, CSF analysis and detailed laboratory investigations.

Targeted single gene analysis using direct Sanger sequencing was performed in three different family members (III.2, III.5, IV.7) and did not reveal any pathogenic mutations in genes causing SPG3A, 4, 6, 7, 8, 10, 13, 31, 33, and SPG42. For SPG3A, SPG4 and SPG31, additionally MLPA (multiplex ligation-dependent probe amplification) assays were performed to exclude copy number variations. All participants included in this study signed written informed consent.

Whole-exome sequencing and validating Sanger sequencing analysis

For whole-exome sequencing, genomic DNA was enriched using the SureSelectXT V5 exome kit (Agilent, Böblingen, Germany) according to the manufacturer's instructions. Sequencing of captured DNA was performed on a HiSeq2500 sequencer (Illumina, San Diego, CA, USA). Reads were mapped against the human reference genome (hg 19 from the UCSC Genome Browser) with the Burrows-Wheeler Aligner (BWA) tool using the MEM algorithm.[13] Variant calling - including small insertions and deletions as well as single nucleotide variants (SNVs) – was performed by VarScan 2.3 (ref.14) and by SAMtools mpileup 0.1.18 with bcftools and

vcfutils.pl..[15] Calls also found in dbSNP (Database of Single Nucleotide Polymorphisms, National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA; Build ID: 137; http://www.ncbi.nlm.nih.gov/SNP/)[16] or the Exome Variant Server database (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, USA; Build ID: 6500; http://evs.gs.washington.edu/EVS/) with an allele frequency >5% were removed. In addition, variants frequently observed in an in-house database produced from the same sequencing technology and enrichment kit were removed (>15%, number of references: 601). Transcript and protein alterations were annotated with NGS-SNP v1.010 (ref. 17) using the ENSEMBL v69 database.[18] Only variants potentially changing the protein sequence were used for further analysis; intronic, UTR and synonymous sequence variations without a predicted splicing defect were removed. Subsequently, the remaining SNVs and insertions or deletions of all three patients were used to find common mutations by a self-developed tool (i.e., mutations that were called in all three patients with identical chromosome, start position, reference allele, and alternative allele).

For Sanger sequencing, genomic DNA was extracted from peripheral blood leukocytes following standard protocols. Exon 6 of the *ERLIN2* gene was amplified by PCR on a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the Ampli Taq Gold® Fast PCR Master Mix (2X) (Applied Biosystems) and flanking intronic primers. Sequences of the primer pair specific to exon 6 of *ERLIN2* (including 155 base pairs of intron 5 and 139 base pairs of intron 6) were as follows: ERLIN2_Ex6_F: 5`-tgaacttcctggtcccgaacgca-3´ and ERLIN2_Ex6_R: 5´-tctccatgaactcctttgtgaccagctc-3´.

Purified PCR products were then sequenced in both directions on a Veriti® 384-Well Cycler (Applied Biosystems) using the Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) and the same primers as for the initial amplification. Finally, the sequencing reactions were analyzed on a 3730xl DNA Analyzer (Applied Biosystems). DNA alignment and sequence variant analysis were carried out using the Sequence Pilot® software (JSI medical systems GmbH, Kippenheim, Germany).

In silico analysis

Putative effects of the detected sequence variants on protein structure and function were evaluated using different *in silico* prediction analysis tools, including PolyPhen-2 [http://genetics.bwh.harvard.edu/pph2], PROVEAN [http://provean.jcvi.org], SIFT [http://sift.jcvi.org], Mutation taster [http://mutationtaster.org], and FATHMM [http://fathmm.biocompute.org.uk].

Conservation scores for the mutated DNA base were evaluated applying the softwares phyloP[19] and GERP (Genomic Evolutionary Rate Profiling).[20]

Results

Clinical presentation

The index patient (IV.7), who originates from Germany, (IV.7) presented to our center for genetic counseling. She reported a positive family history for HSP over three generations, depicted in the pedigree chart in Fig. 1. Patient (IV.7) is the oldest child of the core subfamily. Her brother (IV.8) was obviously healthy. The parents (the affected father III.5 and the unaffected mother) as well as the other members of the extended family did not have a consanguineous relationship. Clinical data of single family members are summarized in Table 1. Of the seven family members who were available for clinical examination, four subjects (II.1, III.2, III.6, IV.7) were diagnosed as definitely affected with HSP, whereas one individual (III.5) with lower-limb hyperreflexia and only mild spastic gait was classified as mildly affected. All other affected members showed slowly progressive spasticity and mild weakness of the lower limbs with onset in adolescence or adulthood (mean age of onset 23.8 years), optionally accompanied by mild reduction of lower-extremity vibration sense reflecting subtle dorsal column affection. Urinary urgency was not encountered. None of the patient had signs of upper limb spasticity, and none showed additional neurological or non-neurological features consistent with a complicated form of HSP. In particular, complicating signs described in autosomal recessive SPG18, which include psychomotor delay, intellectual disability, or fixed joint contractures, were absent. Disease severity assessed by the SPRS reached 4 to 19 points, and disability stages - based on the landmarks of walking ability in the FHSPS - ranged from 1 to 5. As shown in Table 1, age at onset varied greatly among the affected individuals, ranging from 13 up to 46 years. Individuals IV.1, IV.2 and V.1 were not available for clinical assessment and genotyping, but information obtained from other family members suggests that IV.1 is probably affected.

The index patient additionally suffered from migraine without aura and had two generalized epileptic seizures at the age of 18 years. Diagnostic evaluation did not reveal evidence for symptomatic epilepsy, and in 8 years of follow-up no further seizures were recorded under antiepileptic medication with lamotrigin. Of note, intellectual functions of the index patient and brain MR imaging were normal. Since epileptic seizures were not observed in any other affected family member, the possibility remains that the two isolated seizures in this single subject may be coincidental and not part of the HSP phenotype in this family. Interestingly, neoplasms were quite prevalent in the extended family (i.e. leukemia in III.1, hypopharynx carcinoma in III.2, and breast carcinoma in III.3, respectively). However, they are unlikely to be linked to the HSP phenotype in this family, since they occurred also in two family members not affected with HSP, and in the case of the only individual also affected

with HSP (III.2), neoplasia (hypopharynx carcinoma) was presumably associated with extensive abuse of both alcohol and cigarettes.

Additional extensive examinations including brain and spinal MRI, electroneuromyography (ENG/EMG), EEG, CSF analysis and laboratory investigations in two affected family members (III.2 and IV.7) did not reveal any seminal findings. In patient III.2, motor evoked potentials confirmed affection of the corticospinal tract with prolonged central motor conduction times to the legs. In patient IV.7, somatosensory evoked potentials revealed subclinical affection of the dorsal columns, whereas nerve conductions studies were normal. Consistent with uncomplicated HSP, in which cervical and/or thoracic spinal volume loss seems to be the MRI abnormality most commonly observed, mild thinning of the thoracic spinal cord was encountered in proband III.2.

In summary, the phenotype in affected subjects of this family corresponds to an uncomplicated form of HSP with late onset in adolescence or adulthood.

Mutation identification and validation

Whole exome sequencing was performed on genomic DNA of three clinically affected individuals (the index patient, IV.7, her father, III.5, and her grandfather, II.1). In average, 79 million paired reads with a length of 100 bp were produced per exome, with an average coverage of 66 for the enriched target regions, with 97% of the target regions covered more than 10-fold and 82% covered more than 30-fold.

Filtering as described above lead to the identification of only one overlapping variant, located in the *ERLIN2* gene on chromosome 8p11.2 (NCBI Gene ID: 11160; HGNC ID: 1356) and shared by all three affected family members. Subsequent conventional Sanger sequencing (see Fig. 2) confirmed the segregation of this heterozygous missense mutation (c.386G>C, c.DNA 453G>C, g.8060 G>C; p.S129T) with the disease in all affected family members available for genetic analysis (II.1, III.2, III.5, III.6, IV.7; see Fig. 1). Mutation analyses and nomenclature refer to the reference sequences NG_032059.1 (*ERLIN2* genomic DNA sequence), NM_007175.6 (*ERLIN2* transcript variant 1 mRNA sequence) and NP_009106.1 (erlin-2 isoform 1 protein sequence), respectively.

This variant is not listed in dbSNP and in other public databases collecting NGS data, i.e. the databases of the Exome Variant Server (http://evs.gs.washington.edu/EVS/), the 1,000 Genomes project (http://www.1000genomes.org)[21] and the International HapMap project (http://hapmap.ncbi.nlm.nih.gov/).[22] At the protein level, the mutation is predicted to result in a serine to threonine substitution at position 129 (p.S129T) (see Fig. 2), within the SPFH (stomatin-prohibitin-flotillin-HflK/C) domain of erlin-2 (see Fig. 3). This residue

is phylogenetically highly conserved according to phyloP and GERP both in erlin-2 as well as in the homologous protein erlin-1, which share ~70% identity and ~80% similarity at the amino acid level.[23]

In silico analysis

All bioinformatic tools employed for *in silico* analysis consistently predicted pathogenicity of the p.S129T erlin-2 variant, i.e. classified the variant as "deleterious" (PROVEAN), "damaging/affecting protein function" (SIFT), "probably damaging" (PolyPhen-2), "damaging" (FATHMM), and as "disease causing" (Mutation Taster), respectively. Moreover, analysis by GERP and phyloP indicate a very high interspecies sequence conservation of this particular site (prediction scores and corresponding thresholds are given in Fig. 2). Taken together, these results suggest that this variant represents a pathobiologically relevant mutation.

Discussion

We report the identification of a novel heterozygous missense mutation (c.386G>C, p.S129T) in the *ERLIN2* gene, completely segregating with the disease in a German family with a phenotype corresponding to late-onset, pure HSP and, notably, with an autosomal-dominant inheritance pattern. This contrasts fundamentally with earlier reports on *ERLIN2* mutations, found in consanguineous families from the Middle East region with early-onset, severe forms of complicated AR-HSP or juvenile PLS. AR-HSP in these families was commonly characterized by onset in infancy or early childhood and additional complicating features including intellectual disability, speech regression, motor dysfunction, and in particular – as the most prominent and conjunctive attribute – multiple joint contractures [5, 6, 7] (see Table 2).

So far, only one form of HSP typically associated with an autosomal recessive inheritance pattern, namely SPG7, has been described to potentially manifest also as an autosomal dominant disorder.[24, 25, 26] Vice versa, only a few single particular forms of autosomal dominantly inherited HSP have been reported to be transmitted also in a recessive manner, including SPG3A/*ATL1* [22] and just recently SPG72/*REEP2*.[8, 28] As in this study, the corresponding mutations were also identified using whole exome sequencing. Considering that both AR and AD inheritance modes occur in SPG18 and SPG7, other forms of recessively inherited HSP may potentially also manifest as autosomal dominant disorders. Further, the fact that SPG18 and other forms of HSP may present as both dominantly or recessively inherited disorders has significant implications for genetic counseling. This report adds *ERLIN2* to the repertoire of genes known to cause autosomal dominant HSP, which accounts for about 70–80% of all forms of HSP in Western countries. The true prevalence of AD-HSP related to SPG18 has to be determined in further studies.

SPG18 and also the allelic variant juvenile PLS were previously all described as disorders due to homozygous gene mutations, including two frame-shift mutations in exon 11 (2bp insertion with frame-shift)[6] and exon 8 (1bp deletion with frame-shift)[8], respectively, a large deletion with loss of two *ERLIN2* alternative initiation exons along with mislocalization of exon 2 (ref. 5), an intronic splice acceptor site mutation in intron 7 associated with complicated HSP[7] or juvenile PLS[9], and a missense mutation in exon 8 (ref. 8). An overview on the currently known mutational spectrum in the *ERLIN2* gene and clinical features of *ERLIN2*-associated disorders is shown in Table 2. The phenotype of late onset, pure HSP in this family may be either due to a gain-of-function (neomorphic), dominant-negative (antimorphic) or loss-of-function (hypomorphic) effect of the p.S129T mutation, whereas a complete loss of function (nullimorphic mutation), as described in the case of a family with AR-SPG18 (ref. 5), appears unlikely. Obviously, SPG18 does not only occur in the geographical background of the Middle Eastern region, but also in countries of the Western hemisphere. This implies that *ERLIN2* should be considered as a candidate disease-causing gene also in HSP-affected individuals originating from Western countries.

We consider the *ERLIN2* variant (c.386 G>C, p.129T) identified in this study as pathogenic and likely disease causing based on the following reasons:

(i) Sanger sequencing of all affected and unaffected individuals in this family showed complete segregation of this mutation with the disease phenotype.

(ii) The c.386G>C (p.S129T) change is not listed as single nucleotide polymorphism in public databases collecting NGS data (dbSNP, NHLBI Exome variant Server, 1000 genomes project[21], International HapMap project database[22]) nor in an internal-control database of CeGaT (>500 exomes).

(iii) The mutation affects an evolutionary highly conserved amino acid residue localized within the SPFH domain of erlin-2 and was consistently predicted to be damaging or deleterious by several *in silico*-prediction tools.

Reviewing the phenotypic features of all affected members in this family, the missense mutation segregates with a pure form of HSP, characterized by slowly progressive lower limb spasticity, optionally accompanied by mild dorsal column impairment. Age at onset considerably varies among the affected individuals (range: 13 to 46 years). This variation is not unexpected for AD-HSP, since strong inter- und intrafamilial variability regarding age at onset is observed also for other forms of AD-HSP, e.g. SPG4[11], SPG3, and SPG31[29]. In this regard, the obvious difficulty in recognizing the precise time of onset of symptoms that begin insidiously and might go unnoticed for many years should also be considered.

We observed considerable phenotypic variability in this family, with e.g. individual III.5 being only mildly affected (lower-limb hyperreflexia and mild spastic gait) upon neurological examination at age 50, whereas his daughter (IV.7) is definitely and relatively severely affected, with spastic paraplegia starting at age 13. This phenomenon of phenotypic variability, sometimes combined with incomplete or reduced penetrance even at high age, has been described also for other types of AD-HSP, e.g. SPG4[11] and SPG3[30]. Accordingly, autosomal dominantly inherited disease-causing mutations are frequently identified in apparently sporadic patients, e.g. in SPG4[31], SPG3A/SPG4 and SPG31[29]. Overall, the herein described phenotype is virtually indistinguishable from other frequent forms of autosomal dominant pure HSP, which limits the predictability of the genotype based on phenotypical criteria alone.

The pathogenic mechanism of the *ERLIN2* p.S129T mutation identified in this study remains elusive. *ERLIN2* and the highly homologous *ERLIN1* encode lipid raft-associated proteins localized to the endoplasmic reticulum (ER).[32] Erlin-2 and erlin-1, also known as SPFH2 and SPFH1, respectively, belong to a family of mammalian proteins that contain an "SPFH" domain, a ~200 amino acid motif with minor sequence similarities to the proteins stomatin, prohibitin, flotilin, and HflC/K.[32] SPFH proteins share similar properties, including localization to cholesterol-rich, detergent-resistant membranes (DRMs) and assembly into large oligomeric structures.[33] Eukaryotic members of the SPFH protein superfamily are involved in the scaffolding of specific detergent-resistant membrane microdomains with distinct lipid compositions (lipid rafts), suggesting that the SPFH domain may constitute a lipid recognition motif. However, SPFH proteins have distinct subcellular localizations and roles.[32] Erlin-2 has been functionally linked to the ER-associated degradation (ERAD) pathway, a multistep degradative pathway encompassing ubiquitin-proteosome-mediated degradation of ER proteins.[23, 34] Specifically, it plays an important role in the ERAD of activated inositol 1,4,5-trisphosphate receptors (ITPRs) [23, 35, 36, 37] and other substrates such as 3-HMGR (3-hydroxy-3-methylglutaryl-CoA reductase).[38] ITPRs constitute a family of Ca^{2+} release channels in the ER membrane, which regulate numerous cellular processes by generation of local and global Ca^{2+} signals, and 3-HMGR functions as a key enzyme in the biosynthesis of cholesterol.[38] Erlin-2 may act as substrate recognition factor that selects specific or highly restricted groups of proteins for degradation. Moreover, it has been shown that erlins are additional cholesterol binding proteins that are directly involved in regulating the SREBP (sterol regulatory element binding proteins) machinery in the ER, which in turn regulates key transcription factors for genes involved in cholesterol and fatty acid biosynthesis.[39] Erlin-2 appears to have important roles in the core ERAD system, which, as an ER quality control system with selection of substrates for proteolysis, is critical for cellular adaptation to stress and survival.

315 It may influence important signaling systems such as intracellular Ca^{2+} signaling and modulate biosynthesis of
316 cholesterol and lipids.
317 It can be speculated that the c.386G>C, p.S129T mutation identified in this study may alter the secondary
318 structure of erlin-2 in the SPFH domain and thereby reduce the activity or affect the ER membrane localization
319 of erlin-2, which might result in impairment of the ERAD pathway with subsequent accumulation of specific
320 aberrant proteins and/or disturbance of the intracellular calcium signaling system. Impaired ERAD of IP3R may
321 lead to persistent activation of IP3 signaling and channel opening, thus keeping neurons in a state of
322 hyperactivity.[5] In addition, dysregulation of cholesterol/fatty acid biosynthesis may occur. Further protein
323 biochemical and cell biological studies are certainly necessary to characterize and understand the role of the
324 p.S129T mutation more precisely.
325 Interestingly, not only *ERLIN2* but also *ERLIN1* mutations have recently been found to cause AR-HSP
326 (designated SPG62; caused by homozygous mutations in *ERLIN1* and identified in three consanguineous
327 families with overall seven affected individuals that exhibited pure or complicated forms of HSP).[8] This further
328 supports the notion that disturbance of the ERAD system is a potential pathogenic mechanism in HSP, and
329 underscores the hypothesis that individual mutations in distinct genes may converge on specific biological
330 pathways in HSP pathophysiology: ERAD (e.g. *ERLIN2, ERLIN1*) and ER biology/shaping (e.g. *ATL1*, *REEP1*,
331 *RTN2*, and *SPAST*) may constitute such exemplary modules in the pathophysiology of HSP.[40, 8]
332 In line with previous studies, e.g. in families with SPG12/*RTN2* (ref. 41), SPG26/*B4GALNT1* (ref. 42) and
333 several AR-HSP subtypes incl. SPG62/*ERLIN1* (ref. 8), our study demonstrates that whole-exome sequencing is
334 a useful and cost-efficient diagnostic tool in rare and genetically and phenotypically highly heterogeneous
335 diseases. Identifying the responsible gene(s) is clearly highly valuable with respect to exact diagnosis, prognosis,
336 genetic counseling and potentially – in the near future – also for clinical interventional studies of etiology-based
337 therapies.
338 Our study supports the notion that distinct SPG genes may cause a phenotypic spectrum of motoneuron
339 diseases[43], in the case of *ERLIN2* encompassing pure HSP, complicated HSP and juvenile PLS.[9] Other examples
340 of SPG genes associated with more than one form of motor neuron disorder represent SPG6/*NIPA1*, causing
341 both pure HSP and ALS[44], SPG11/*KIAA1840* causing both complicated HSP and juvenile ALS[45], SPG17/*BSCL2*
342 causing complicated HSP, Charcot-Marie-Tooth disease type 2 (CMT2), and distal hereditary motor neuropathy
343 type V (dHMN-V)[46,] and SPG31/*REEP1* causing both pure HSP and dHMN-V.[47, 48] In many of these examples,
344 expansion of the phenotypic spectrum was facilitated by whole-exome sequencing.

In summary, a novel *ERLIN2* missense mutation segregating with a late-onset, pure HSP phenotype and an autosomal dominant inheritance pattern was identified in this study, expanding the mutational and phenotypic spectrum of SPG18. The prevalence of AD-SPG18/*ERLIN2*-associated HSP in the Western hemisphere remains to be determined in further studies. Nevertheless, we suggest to include genetic testing for *ERLIN2*/SPG18 mutations in the diagnostic algorithm also for cases presenting with late-onset, pure, autosomal-dominant and also sporadic forms of HSP.

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Authors` contributions

Study concept and design: AD, FR, CK; Acquisition of clinical data and blood sample collection: AD, FR, CK; Exome sequencing and mutational analysis: CF, SK, SB; Analysis and interpretation of data: AD, FR, CK, CF, SB; Drafting of the manuscript: AD, FR; Critical revision of the manuscript for important intellectual content: all authors. All authors gave final approval for the manuscript to be published.

Conflict of interest

The authors declare that they have no conflict of interest.

Titles and legends to Figures

Fig. 1 Pedigree of the family with autosomal dominant HSP linked to SPG18

Black filled symbols denote affected, white symbols unaffected family members (squares: men, circles: women; slashed symbols represent deceased individuals). Roman numerals next to each symbol represent the generation, Arabic numerals identify individuals. The index patient (IV.7) is indicated by a black arrow. Whole-exome sequencing was performed in three subjects indicated by red stars (II.2, III.5, IV.7). Sanger sequencing for validation was performed in all subjects indicated by red squares with either a plus (confirmation of mutation) or a minus sign (mutation not present) within the square (II.1, III.2, III.4, III.5, III.6, IV.3, IV.7, IV.8), demonstrating complete segregation of the novel *ERLIN2* missense mutation c.386G>C, p.S129T with the disease.

Fig. 2 Novel mutation in *ERLIN2* (c.386G>C, p.S129T)

a Chromatograms showing the novel *ERLIN2* mutation c.386G>C in the heterozygous state, resulting in amino acid substitution of serine with threonine in the index patient IV.7, and the wild type sequence in the unaffected subject III.4.
b Alignment of the corresponding protein sequence surrounding amino acid position 129 in various species. Amino acid residues deviant from the wild type human sequence are colored in yellow.
c *In silico* predictions on protein structure/function and predictions on grade of conservation.

Fig. 3 Schematic representation of all ERLIN2 transcript variants, the protein encoded by the largest isoform, and the site of the p.S129T mutation (adapted after Yıldırım et al. 2011)

a Transcript variants of ERLIN2. The coding regions are shown in squares.
b Basic structure and domain organization of the erlin-2 protein (isoform 1). Domains and distinct motifs are differentially colored, with corresponding amino acid number of predicted boundaries on top. The single pass type II membrane protein (amino acid residues 1 to 339) is composed of a short, N-terminal cytosolic domain (CD; residues 1-3), a short transmembrane domain (TMD; residues 4-24) spanning the ER membrane, and a larger luminal domain (residues 25-339) containing the SPFH domain (residues 22-226), the oligomerization domain (residues 228-300) and a short hydrophobic patch containing a phenylalanine residue at position 305 (residues 301-306) (Hoegg et al. 2009).
c The site of the novel mutation (c.386G>C, S129T) identified in this study is indicated on mRNA and protein, respectively, by a blue arrow.

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Individual	Sex	Age at last examination	Age at onset	FHSPS [a] and SPRS [b]	Spastic gait	Increased tone in LL	Hyperreflexia in LL	Weakness in LL	Extensor plantar response	Additional features/ remarks
II.1		76	30	FHSPS: 5 SPRS: N.A.	N.A.	+++	++	++	+	- Stroke in late adulthood with resulting dysarthria - death at age 77
III.1	M	N.A.	N.A.							- death at age 33 (leukemia)
III.2	M	45	14	FHSPS: 4 SPRS: 19	+++	++	++	++	+	- requirement of intrathecal baclofen therapy at age 46 - Hypertonia, COPD, Sensible PNP (associated with alcohol abuse) - death at age 55 (hypopharynx carcinoma)
III.3	F	N.A.	-							- death at age 53 (breast carcinoma)
III.4	F	40	-	-	-	-	-	-	-	-
III.5	M	39	16	FHSPS: 1 SPRS: 4	+	-	+	-	-	-
III.6	F	48	46	FHSPS: 2 SPRS: 9	++	+	+++	+	+	-
IV.3	F	34	-	-	-	-	-	-	-	-
IV.7	F	25	13	FHSPS: 3 SPRS: 12	+++	++	+++	+	+	- Migraine without aura - 2 occasional seizures - Allergic asthma
IV.8	M	22	-	-	-	-	-	-	-	-

Table 1 Summary of clinical features observed in members of the German family with AD-HSP under study.

[a] Gait disability stages according to the FHSPS (Functional Hereditary Spastic paraplegia Rating Scale): 1, no mobility problems or slight stiffness of the legs; 2, moderate gait stiffness; 3, problems running, but able to walk alone; 4, problems in walking; 5, wheelchair-bound.[11, 12]

[b] SPRS (Spastic Paraplegia Rating Scale): Range 0-52. (ref. 10)

‘‘-“ indicates absent, ‘‘+’’ indicates mild, ‘‘++’’ indicates moderate, and ‘‘+++’’ indicates severe.
‘‘LL’’ denotes lower limbs. “N.A.” denotes not applicable. COPD: chronic obstructive pulmonary disease; PNP: polyneuropathy.

A8

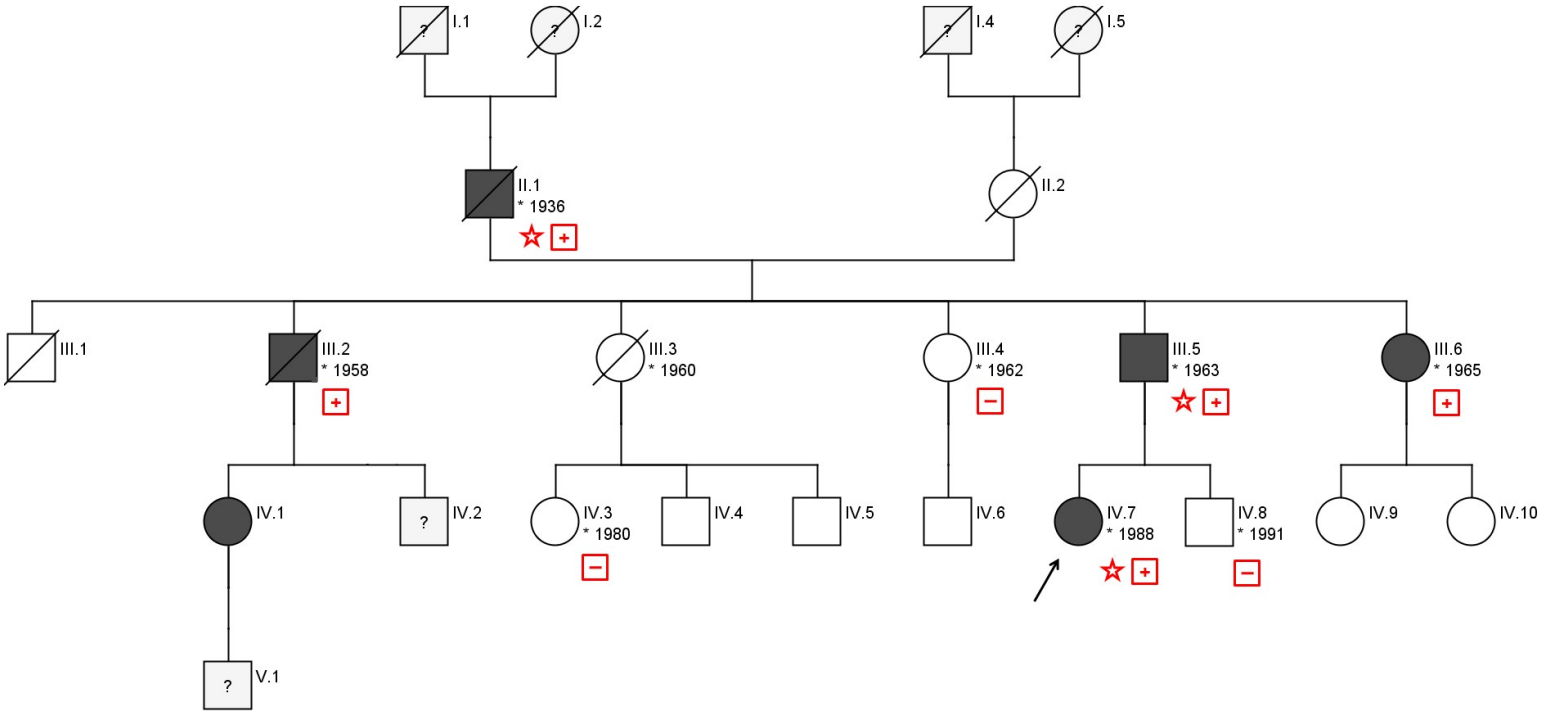
Mutation [a] - Mutation type - NT change,AA change - Exon/ Intron	**Family** - Affected members - Consanguinity	**Origin**	**Phenotype** specific clinical features, additional paraclinic/imaging findings	**Age at onset**	**Reference**
- **Large nullimorphic deletion (2 bp):** - Loss of 2 alternative initiation exons along with mislocalization of exon 2, causing a nullimorphic allele - breakpoint of del immediately upstream of exon 2	- 1 family, 5 affected members - Consang.	Saudi Arabia	**Severe complex HSP:** progressively worsening spasticity, ascending from lower to upper extremities + - intellectual disability - expressive language regression/ aphasia - one patient with epilepsy (atypical absence epilepsy) - questionable: congenital hip dislocation (one patient)	**Early onset:** during first year	5
- **Missense mutation: homozyg** - c.528C>G, p.W176C - exon 8	- "family 1334"	N.A.	**complex HSP:** (details N.A.)	N.A.	8
- **Splice acceptor site mutation: homozyg** - c.499-1 G>T, p.Q169LfsX4 - Intron 7	- 1 family; 2 affected siblings (2/4 children) - Consang. (parents: first degree cousins)	Saudi Arabia	**complex HSP:** spasticity of the lower extremities with significant weakness, upper extremities mildly hypertonic + - cognitive involvement, developmental delay - speech involvement (dysarthria, speech regression) - motor involvement - one patient with multiple contractures	**Early onset:** during first year	7
- **Splice acceptor site mutation: homozyg** - c.499-1G>T - Intron 7	- 1 family, total of 11 siblings, 4 affected individuals (3 m, 1 f) - Consang.	Central region of Arabian Peninsula	**Juvenile HSP** : Pseudobulbar palsy, slow tongue movement; mild to moderate distal pyramidal weakness; muscle bulk moderately wasted distally; muscle tone increased in both axial and limb muscles, with exaggerated tendon reflexes and plantar response + - disrupted smooth pursuit by large saccadic intrusions - speech and articulation development initially normal, then regression after the age of 2 years - dysmorphic signs: High arched palate and narrow upper jaw - kyphosis and scoliosis around the age of 13-14 years # brain and spinal MRI: normal	**Early onset:** around the age of 8 months	9
- **Frameshift mutation: homozyg** - c.549delC, p.x? - exon 8	- "family 1055"	N.A.	**complex HSP:** (details N.A.)	N.A.	8
Frameshift mutation: homozyg - c.812_813insAC, p.N272PfsX4 - exon 11	- 12 affected individuals from 5 core families (one village with in total 27 affected individuals) - Consang. (families founded by 5 immigrant siblings and their consang. spouses)	Eastern Turkey	**complex HSP with DD (intellectual disability motor dysfunction and joint contractures**): Spasticity + - Severe ID (only not-verbal communication possible) - Severe motor dysfunction (arrest and regression in motor functions, all affected could not sit unsupported or walk) - multiple joint contractures resulting in specific fixed posture - some: with febrile convulsions (9 individuals) - some: dysmorphic signs facio-cranial (i.e. large mouth and tongue) # CCT (one patient) or cMRI (two patients): normal	**Early onset:** 6 months to 2 years	6
- **possible linked to *ERLIN2*** [b]**:** HSP linked to chr. 8p12–p11.21 (locus harbors *DDHD2* and *ERLIN2* gene)	- 1 family, total of 7 children, 3 affected - Consang. (parents: first degree cousins)	Oman	**complex HSP:** spastic paraplegia of the lower limbs + - Epilepsy (frequent generalized seizures) in 2 of 3 affected children - normal mental development # CCT scans (all 3 patients): normal	**Early onset:** 4-6 years	49
- **Missense mutation: heterozyg** c.386G>C, p.S129T - exon 6	- 1 family with 6 affected individuals - No Consang.	Germany	**Pure complex AD HSP**	**Variable onset** (adolescence – adult-hood): 13-46 years	This report

Table 2 Reported mutations and clinical characteristics in *ERLIN2*-associated disorders (SPG18 and juvenile PLS)

[a] Numbering of mutated nucleotides is based on NCBI Reference Sequences (*ERLIN2* genomic DNA: accession number NG_032059.1, *ERLIN2* mRNA (transcript variant 1): accession number NM_007175.6, erlin-2 protein (isoform 1): accession number NP_009106.1). Exon numbering: exon containing start codon = exon 2.

[b] In the study of Al-Yahyaee et al.[49], two unrelated Omani families with distinct AR-HSP forms were mapped to a candidate disease locus on chromosome 8p12-p11.21. One family was later identified to suffer from SPG54, caused by a mutation in the *DDHD2* gene within the candidate locus[50], whereas the other family (with epilepsy in 2 of 3 affected members) has so far – to the best of our knowledge – not been linked to a known HSP gene, but *ERLIN2* may be a candidate gene within this locus.

Abbrevations: AA, amino acid; CCT, cranial computional tomography; chr., chromosome; cMRI, cranial magnetic resonance imaging; consang., consanguinity; Heterozyg., heterozygous; homozyg., homozygous; HSP, hereditary spastic paraplegia; N.A., not available; NT, Nucleotide; PLS, primary lateral sclerosis.


I.1
I.2
I.4
I.5
II.1
* 1936
II.2
III.1
III.2
* 1958
III.3
* 1960
III.4
* 1962
III.5
* 1963
III.6
* 1965
IV.1
IV.2
IV.3
* 1980
IV.4
IV.5
IV.6
IV.7
* 1988
IV.8
* 1991
IV.9
IV.10
V.1

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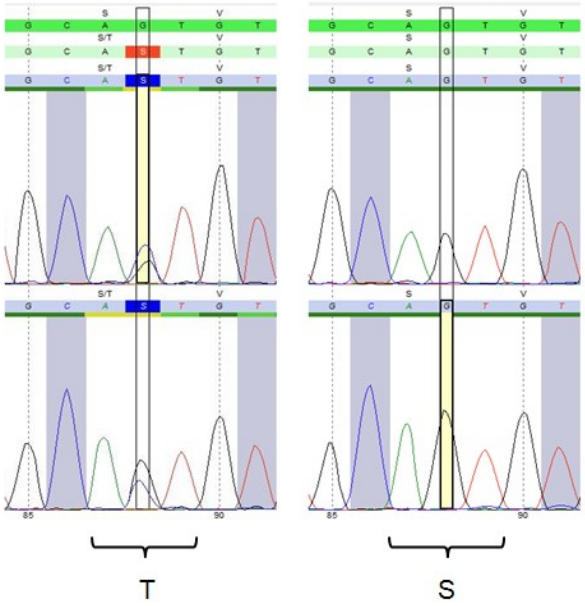
a



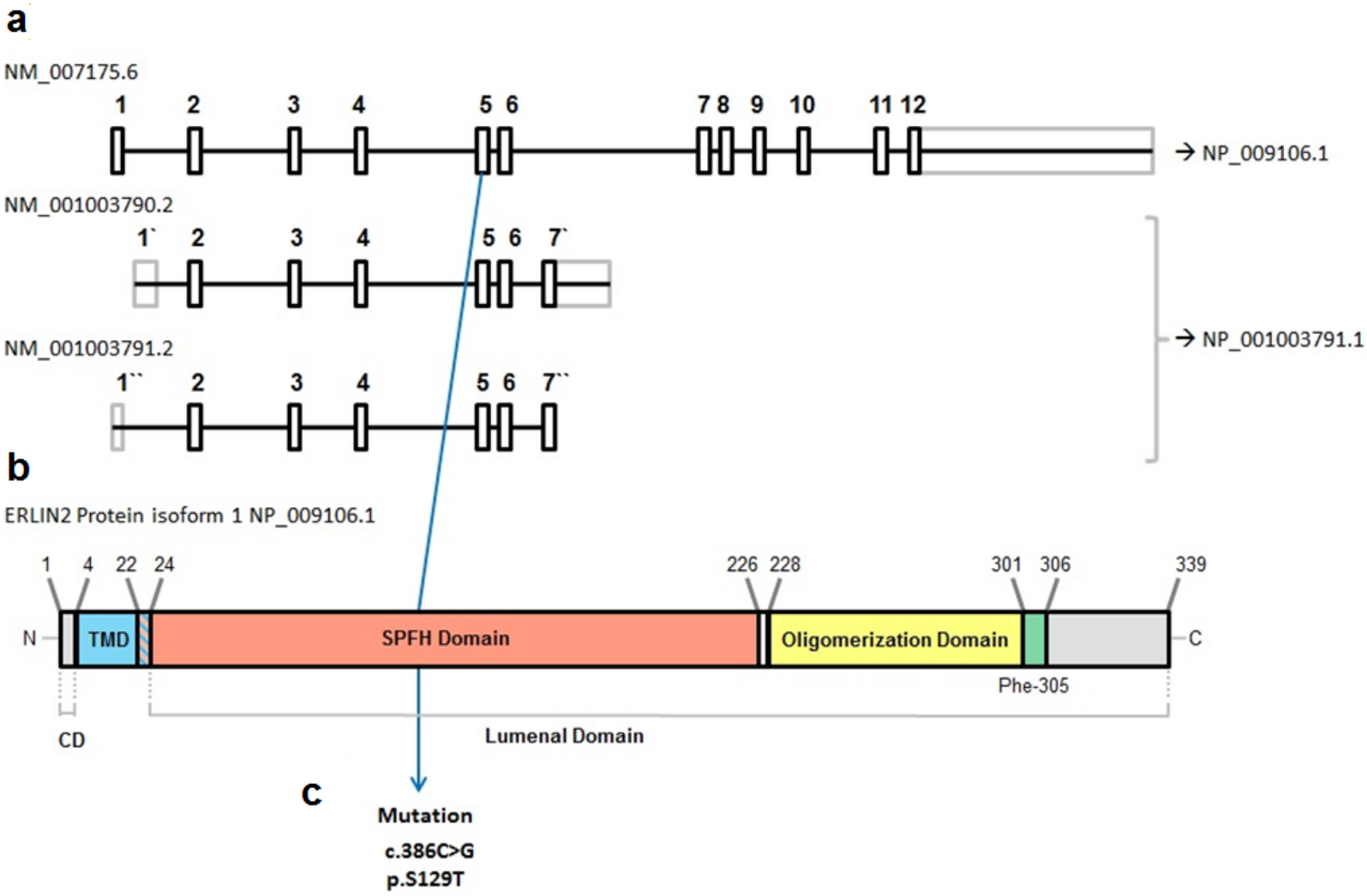
b

c.386C>G
p.S129T

Species	Accession	119		139
Human [Homo sapiens]	NP_009106.1	... K I H H E L N Q F C	S	V H T L Q E V Y I E ...
Human: Mutated	--	... K I H H E L N Q F C	T	V H T L Q E V Y I E ...
Chimpanzee [Pantroglodytes]	XP_001169738.1	... K I H H E L N Q F C	S	V H T L Q E V Y I E ...
Mouse [Mus musculus]	NP_705820.1	... K I H H E L N Q F C	S	V H T L Q E V Y I E ...
Rat [Rattus norvegicus]	NP_001099558.1	... K I H H E L N Q F C	S	V H T L Q E V Y I E ...
Zebrafish [Danio rerio]	NP_001121887.1	... K V H H E L N Q F C	S	V H T L Q D V Y I G ...
Worm [Caenorhabditis elegans]	NP_502339.1	... K V H H E V N Q F C	S	V H T L Q E V Y I E ...

c

	Prediction for S129T (pos 8:37,602,176)	Score	Threshold
Prediction of functional effect			
Provean	**Deleterious**	**-2.97**	< -2.5
SIFT	**Damaging**	**0.002**	< 0.05
PolyPhen2	**Probably damaging**	**0.979 (HDIV)**	> 0.957 (HDIV)
		0.973 (HVAR)	> 0.909 (HVAR)
FATHMM	**Damaging**	**-3.24**	< -1.5
Mutation Taster	**Disease causing**	-	-
Prediction grade of Conservation			
phyloP	**Highly conserved**	**9.869**	
GERP	**Highly conserved**	**6.16**	-12.3 to 6.17 (with 6.17 being the most conserved)


a
NM_007175.6
1 2 3 4 5 6 7 8 9 10 11 12
→ NP_009106.1
NM_001003790.2
1` 2 3 4 5 6 7`
NM_001003791.2
1`` 2 3 4 5 6 7``
→ NP_001003791.1
b
ERLIN2 Protein isoform 1 NP_009106.1
1 4 22 24 226 228 301 306 339
N
TMD
SPFH Domain
Oligomerization Domain
C
Phe-305
CD
Lumenal Domain
c
Mutation
c.386C>G
p.S129T

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ORIGINAL RESEARCH
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Rare Variants in Neurodegeneration Associated Genes Revealed by Targeted Panel Sequencing in a German ALS Cohort

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Amyotrophic lateral sclerosis (ALS) is a progressive fatal multisystemic neurodegenerative disorder caused by preferential degeneration of upper and lower motor neurons. To further delineate the genetic architecture of the disease, we used comprehensive panel sequencing in a cohort of 80 German ALS patients. The panel covered 39 confirmed ALS genes and candidate genes, as well as 238 genes associated with other entities of the neurodegenerative disease spectrum. In addition, we performed repeat length analysis for *C9orf72*. Our aim was to (1) identify potentially disease-causing variants, to (2) assess a proposed model of polygenic inheritance in ALS and to (3) connect ALS with other neurodegenerative entities. We identified 79 rare potentially pathogenic variants in 27 ALS associated genes in familial and sporadic cases. Five patients had pathogenic *C9orf72* repeat expansions, a further four patients harbored intermediate length repeat expansions. Our findings demonstrate that a genetic background of the disease can actually be found in a large proportion of seemingly sporadic cases and that it is not limited to putative most frequently affected genes such as *C9orf72* or *SOD1*. Assessing the polygenic nature of ALS, we identified 15 patients carrying at least two rare potentially pathogenic variants in ALS associated genes including pathogenic or intermediate *C9orf72* repeat expansions. Multiple variants might influence severity or duration of disease or could account for intrafamilial phenotypic variability or reduced penetrance. However, we could not observe a correlation with age of onset in this study. We further detected potentially pathogenic variants in other neurodegeneration associated genes in 12 patients, supporting the hypothesis of common pathways in neurodegenerative diseases and linking ALS to other entities of the neurodegenerative spectrum. Most interestingly we found variants in *GBE1* and *SPG7* which might represent differential diagnoses. Based

A9

on our findings, we recommend two-staged genetic testing for ALS in Germany in patients with familial and sporadic ALS, comprising *C9orf72* repeat analysis followed by comprehensive panel sequencing including differential diagnoses that impair motor neuron function to meet the complexity of ALS genetics.

Keywords: amyotrophic lateral sclerosis, neurodegeneration, next generation sequencing, genetic heterogeneity, polygenic inheritance

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating multisystemic neurodegenerative disorder characterized by degeneration of upper and lower motor neurons in the motor cortex, brain stem, and spinal cord (Peters et al., 2015). ALS can be inherited in an autosomal dominant, autosomal recessive or X-linked manner. About 10% of cases are considered as being familial (fALS), whereas the remaining 90% seem to occur sporadically (sALS) with no family history of ALS. Since the first discovery of *SOD1* mutations being causative for ALS1 in 1993 (Rosen et al., 1993), researchers all over the world have made great effort to further delineate the genetic basis underlying ALS. Today, more than 30 confirmed major disease genes are listed by the Amyotrophic Lateral Sclerosis Online genetics Database (ALSoD[1]), the most frequently affected being *C9orf72* (40% fALS, 5–6% sALS; pathogenic repeat expansion in the non-coding region between exons 1a and 1b, detection by repeat analysis), *SOD1* (20% fALS, 3% sALS), *FUS* (5% fALS, <1% sALS) and *TARDBP* (3% fALS, 2% sALS) (Abel et al., 2012; Su et al., 2014).

Screening of the known ALS genes identifies pathogenic mutations in more than 60% of fALS cases. However, the same genes that can be affected in fALS can also be found mutated in sporadic cases, e.g., due to incomplete penetrance, false paternity, recessive inheritance or *de novo* mutations (Su et al., 2014). Mutations in disease genes affect different molecular pathways which promote motor neuron degeneration and include protein misfolding and subsequent aggregation, mitochondrial dysfunction and oxidative stress, impaired RNA processing, glutamate excitotoxicity and impaired axonal transport (Redler and Dokholyan, 2012; Shaw, 2005). These findings provided fundamental insight into basic underlying pathomechanisms and additionally linked ALS to other disease entities like frontotemporal dementia (FTD) or hereditary spastic paraplegia (HSP).

With the application of genome-wide association studies (GWASs) and high throughput sequencing technologies (next generation sequencing, NGS), a large number of additional disease genes, disease modifiers, and risk factors have been identified especially in sALS. GWASs suggest that genetic factors might contribute to a minimum of 23% of disease risk, whereupon such factors do not necessarily have to be directly causative but instead may act as risk factors or disease modifiers (e.g., age of onset, disease progression) in the interplay with environmental and stochastic factors (Renton et al., 2014; Marangi and Traynor, 2015). Numerous GWASs have been published which showed associations of various loci with ALS containing potential risk genes such as *FGGY*, *ITPR2* and *UNC13A* (Marangi and Traynor, 2015) but until now, causative variants in most of these genes have not been identified. As GWASs are based on the "common disease – common variant" hypothesis and odds ratios associated with risk alleles are usually low, they are solely suitable for the identification of common disease modifiers with low effect size in complex disorders rather than rare causative variants with large effect sizes. By contrast, NGS represents a powerful, groundbreaking approach to detect rare variants with moderate or high penetrance in Mendelian diseases without having access to large pedigrees (He et al., 2014). ALS and other neurodegenerative diseases which are characterized by great genetic heterogeneity and sometimes overlapping symptoms or even atypical phenotypes benefit to a great extent from NGS and the possibility to analyze all genes implicated in the disease in one approach. During the last years, the use of NGS encompassed and considerably increased the number of identified disease genes and risk factors for ALS, generating further insight into underlying pathomechanisms at the same time. One example is the recent discovery of the mitochondrial protein CHCHD10 as being implicated in ALS which for the first time proves a direct impact of mitochondria in the pathogenesis of the disease, a result obtained by exome sequencing in several families affected by ALS (e.g., Bannwarth et al., 2014; Müller et al., 2014; Kurzwelly et al., 2015). As sequencing costs and turnaround times substantially decreased during the last years, the broad application of NGS has triggered a fundamental shift not only in clinical genetics but also in research on rare heritable diseases. Additionally, by the analysis of large numbers of genes in parallel, it has become evident that some patients carry potentially pathogenic variants in genes that are associated with other entities of the neurodegenerative spectrum. Besides this, one emerging theme in ALS genetics is the presumption that ALS might be a complex disease. This view arises mainly from the observation of reduced penetrance in pedigrees affected by fALS and the partially missing heritability in sporadic cases (van Blitterswijk et al., 2012; He et al., 2014). In recent studies, the authors applied NGS to identify patients who carried pathogenic or potentially pathogenic variants in more than one disease gene with frequencies ranging from 1.6% to 31.7% in fALS and sALS cohorts (van Blitterswijk et al., 2012; Kenna et al., 2013; Cady et al., 2015). However, these studies additionally point out that the genetic basis underlying ALS in cohorts of different European countries and the US differs due to founder effects and thus should not be assumed to be homogeneous.

[1]http://alsod.iop.kcl.ac.uk/home.aspx

Here we hypothesize that ALS is caused by polygenic contributions from many disease-causing or disease-modifying gene variants which encompass not only known ALS genes but also other genes from the neurodegenerative disease spectrum. To investigate this hypothesis, we used a high-coverage targeted high-throughput sequencing approach to detect variants in 39 ALS associated genes as well as 238 additional genes that are linked to other neurodegenerative diseases in a German cohort of 80 clinically well characterized ALS patients. We aim at identifying known causative mutations and novel variants, to report on patients who carry multiple potentially disease causing variants or variants in genes which are implicated not only in ALS, but also in other neurodegenerative disorders. To our knowledge, this is the first report on extensive genetic screening in a German ALS cohort including not only confirmed ALS genes but also possible candidate genes, modifiers and risk factors to assess the great genetic heterogeneity of ALS in Germany.

MATERIALS AND METHODS

Study Participants

Our cohort includes 80 unrelated clinically diagnosed ALS patients (55% male, 45% female; 7.5% familial, 92.5% sporadic; 82.5% ALS, 6.25% ALS-FTD, 2.5% flail leg, 2.5% flail arm, 6.25% primary lateral sclerosis (PLS)). Mean age of disease onset was 60.1 years (range 29–88 years). Patients were recruited consecutively in ALS outpatient clinics at the university hospitals Rostock and Bochum (Germany). Relationship was excluded by evaluation of family history. Only one affected individual per family was included in this study and there was no evidence of relationship between any study participants. Informed written consent was obtained from all participants. The study was approved by the local medical ethics committee of Rostock University (A2009-10 and A2011-56) and conducted in accordance with the Declaration of Helsinki.

DNA Extraction

Genomic DNA was extracted from EDTA blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

C9orf72 Repeat Analysis

All subjects were screened for a pathological repeat expansion in the *C9orf72* gene (GenBank NM_018325.3, NM_145005.5) using fragment length analysis of fluorescence labeled PCR products as repeat expansions cannot be detected by NGS (method according to DeJesus-Hernandez et al., 2011). Based on a repeat primed PCR we determined the size of GGGGCC repeats (method according to Renton et al., 2011). Repeat lengths of $\geq$ 30 units were considered as being pathogenic, whereas repeat lengths of 20 to 29 units are considered as intermediate.

Targeted Resequencing

Genomic DNA was enriched using a custom design Agilent SureSelect in solution kit. The design of our diagnostic panel for neurodegenerative diseases (277 genes in total) included 14 genes which were classified as disease genes when this study was initiated, 25 putative candidate genes, modifiers, and risk factors identified by literature research as being most presumably implicated in ALS (e.g., by GWAS, experimental evidence, or connected pathways; **Table 1**), as well as 238 genes associated with other neurodegenerative diseases (for example genes associated with FTD, HSP and others; see Supplementary Data, 763 kb in total). Sequencing was performed using barcoded libraries on the SOLiD 5500xl platform according to the manufacturer's instructions (Fragment Library Preparation 5500 Series SOLiD^{TM} Systems, User Guide, Applied Biosystems by Life Technology). Approximately 2.3 million on target reads were generated per sample and the mean coverage on target was 184.2 sequencing reads with a mean mapping quality of 85.3. On average 89.4% of bases were covered by $\geq$10 reads/base per sample. The primary data analysis was performed using Lifescope (versions v2.5-r0 and v2.5-r2.5.1).

Variant Filtering

Only variants (SNVs/small indels) with a minor allele frequency (MAF) of $\leq$1% in coding and flanking intronic regions ($\pm$8 base pairs) and the UTR regions were evaluated. Known disease causing mutations which are listed in the HGMD database were evaluated in coding and flanking intronic regions up to $\pm$30 base pairs and up to a MAF of $\leq$5%. Population frequencies are adapted from the following databases: 1000 Genomes, dbSNP, Exome Variant Server, ExAC and an internal database. Our quality criteria required coverage of $\geq$10 quality reads per base and a novel allele frequency (NAF) of $\geq$0.3. Detected variants were assessed based on their MAF, current literature and widely used Online databases [e.g., OMIM (McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD, USA), HGMD (Stenson et al., 2014), Uniprot (UniProt Consortium, 2015), locus or disease specific databases] and prediction tools [MutationTaster (Schwarz et al., 2014), PolyPhen2 (Adzhubei et al., 2010), SIFT (Choi et al., 2012), NetGene2 Server (Brunak et al., 1991) and Splice Site Prediction by Neural Network (Reese et al., 1997)].

Comparison of Observed Frequencies

We compared the observed frequencies of affected genes in ALS cohorts from the US (Couthouis et al., 2014), Ireland (Kenna et al., 2013), Italy (Chiò et al., 2012) and Great Britain (Morgan et al., 2015) with detected frequencies in our cohort.

Generation of a Protein–Protein Interaction Network

To visually link candidate genes and possible modifiers to ALS, and to put them in relation to each another and to confirmed ALS genes, we created a protein–protein interaction network containing 21 disease genes and 13 candidate genes, possible

TABLE 1 | Genes analyzed in this study.

Gene	Transcript	Genetic subtype
SOD1	NM_000454.4	ALS1
ALS2	NM_020919.3	ALS2
SETX	NM_015046.5	ALS4
SPG11	NM_025137.3	ALS5
FUS	NM_004960.3	ALS6
VAPB	NM_004738.4	ALS8
ANG	NM_001145.4	ALS9
TARDBP	NM_007375.3	ALS10
FIG4	NM_014845.5	ALS11
OPTN	NM_021980.4	ALS12
ATXN2	NM_002973.3	ALS13
VCP	NM_007126.3	ALS14
CHMP2B	NM_014043.3	ALS17
C9orf72	NM_018325.3	FTDALS1
APEX1	NM_001641.3	
ATXN1	NM_000332.2	
CCS	NM_005125.1	
DAO	NM_001917.4	
DCTN1	NM_004082.4	
DPP6	NM_001936.4	
FGGY	NM_001113411.1	
GLE1	NM_001003722.1	
GRN	NM_002087.2	
HEXA	NM_000520.4	
HFE	NM_000410.3	
ITPR2	NM_002223.2	
KIFAP3	NM_014970.3	
LIF	NM_002309.4	
NAIP	NM_004536.2	
NEFH	NM_021076.3	
PON1	NM_000446.5	
PON2	NM_000305.2	
PON3	NM_000940.2	
RNF19A	NM_183419.3	
SLC1A2	NM_004171.3	
SPAST	NM_014946.3	
UNC13A	NM_001080421.2	
VEGFA	NM_001025366.2	
VPS54	NM_001005739.1	

The top 14 genes were classified as disease genes when this study was initiated; a further 25 candidate genes, modifiers and risk factors were also included. Gene names are HGNC symbols, transcripts are identified by RefSeq accessions.

risk factors, and modifiers covered by our sequencing panel (**Figure 2**). The protein-protein interaction network was created using the STRING database v10[2] by searching for multiple proteins: ALS2, ANG, ATXN1, ATXN2, C9orf72, CHCHD10, CHMP2B, DPP6, ERBB4, FGGY, FIG4, FUS, GBE1, GLE1, GRN, HNRNPA1, ITPR2, KIFAP3, MATR3, NEFH, OPTN, PFN1, PON3, SETX, SIGMAR1, SLC2A1, SOD1, SPG11, SPG7, TARDBP, UBQLN2, UNC13A, VAPB, VCP. Standard settings were used, network edges set to show confidence, and structural previews inside network bubbles were disabled.

[2]http://string-db.org/

RESULTS

Identification of Variants in ALS Associated Genes

By analyzing 39 ALS associated genes (**Table 1**), we were able to detect 79 rare variants (European–American MAF $\leq$ 1% in dbSNP, EVS or ExAC) in 27 genes which passed defined filter criteria (see Variant Filtering) and manual assessment in the Integrated Genome Viewer (IGV, v2.1.28 rev release 175, Robinson et al., 2011; see **Table 2**). Of these, 34 variants have been published previously whereas 45 have not been described before and therefore are considered as being novel. Excluding synonymous substitutions, we identified 54 rare variants in 23 male and 25 female patients (48 patients representing 60% of our cohort). We found that 20 patients of whom 95% (19 out of 20 patients) are considered as sporadic cases carry variants in 14 known disease genes. Additionally we identified variants in candidate genes, modifiers or risk factors in 28 patients (see **Figure 1**).

Pathogenic repeat expansions in the *C9orf72* gene were identified in five (6.25%) sporadic patients (mean age of onset: 67.6 years, range 49–76 years). Two of these patients carried additional variants in *FIG4* and *UNC13A* (pat #10), and *ITPR2* (pat #373), respectively (see **Table 3**). Furthermore, we identified four patients carrying intermediate length repeat expansions (mean age of onset: 57 years, range 40–68 years). Of these, two individuals carried additional missense and splice variants in *ALS2* and *UNC13A* (pat #26), and *SPG11* (pat #729) respectively (see **Table 2**). Given the size of this sample, the remarkable difference in mean age of onset between the patients with intermediate length expansions and carriers of pathogenic repeat expansions is not statistically significant ($p = 0.11$, Wilcoxon–Mann–Whitney test).

By focusing on candidate genes, modifiers, and risk factors, one interesting finding is the identification of four missense variants in the *GRN* gene (see **Table 2**). Of these variants, three have already been described as being probably benign in FTD cases (p.T182M), of unknown clinical relevance in FTD and progressive non-fluent aphasia (p.A324T), or as being potentially pathogenic in FTD spectrum disease (p.V77I), respectively (Guerreiro et al., 2008; Pickering-Brown et al., 2008; Yu et al., 2010). Besides this, we detected seven missense variants in the *ITPR2* gene which was linked to ALS by several GWASs in the past (van Es et al., 2007), eight variants in *FGGY,* and three variants in *UNC13A,* as well as variants in *ATXN1*, *DPP6*, *GLE1*, *KIFAP3*, *NEFH*, *PON3* and *SLC1A2* (see **Table 2**).

Co-occurrence of Variants in ALS Associated Genes

Earlier studies supported a complex genetic basis for ALS, which is also supported by protein–protein interactions between known ALS-associated genes, candidate genes, risk factors, and possible

TABLE 2 | Identified variants in ALS associated genes.

Gene	cDNA	Protein	Zygosity	MAF_EA (%)	MAF (%) in this study	dbSNP	Pat-ID	Gender	Subtype	AAO (years)	Reference	MT	PolyPhen2	SIFT	NG2	NN
ALS disease genes																
ALS2	c.4119A > G	p.I1373M	het	0.52	0.63	rs61757691	#422	f	sALS	61	Kenna et al., 2013	Disease causing	benign	tolerated	–	–
ALS2	c.1816-8C > T	p.?	het	0.25	1.25	rs185911369	#26**	m	PLS	66	–	Polymorphism	–	–	no effect	no effect
ALS2	c.1816-8C > T	p.?	het	0.25	1.25	rs185911369	#524	f	sALS	60	–	Polymorphism	–	–	no effect	no effect
ALS2	c.1127_1129 delAAG	p.E375del	het	–	0.63	–	#45	f	sPLS	43	–	Disease causing	–	–	–	–
ATXN2	c.2049A > T	p.L683F	het	–	0.63	–	#34	f	ALS-FTD	54	–	Disease causing	possibly damaging	damaging	–	–
C9orf72	c.956C > A	p.P319Q	het	–	0.63	–	#37a	m	sALS	50	–	Disease causing	probably damaging	tolerated	–	–
FIG4	c.1940A > G	p.Y647C	het	0.01	0.63	rs150301327	#10*	m	sALS	71	Chow et al., 2009	Disease causing	benign	damaging	–	–
FIG4	c.1910C > A	p.P637Q	het	–	0.63	–	#44	f	ALS	88	–	Polymorphism	benign	tolerated	–	–
SETX	c.3229G > A	p.D1077N	het	0.14	0.63	rs145097270	#571	m	sALS	49	Kenna et al., 2013	Polymorphism	possibly damaging	damaging	–	–
SETX	c.7358A > G	p.K2453R	het	–	0.63	–	#29	f	sALS	73	–	Polymorphism	benign	tolerated	–	–
SPG11	c.6950G > A	p.G2317D	het	<0.01	0.63	rs79186522	#23	f	flail leg	69	–	Polymorphism	benign	tolerated	–	–
SPG11	c.5381T > C	p.L1794P	het	<0.01	0.63	rs201689565	#729**	m	sALS	40	–	Disease causing	probably damaging	damaging	–	–
SPG11	c.3577A > G	p.I1193V	het	0	0.63	–	#747	f	sALS	69	–	Polymorphism	benign	tolerated	–	–
TARDBP	c.931A > G	p.M311V	het	–	0.63	rs80356725	#741	f	sALS	64	Lemmens et al., 2009	Disease causing	benign	tolerated	–	–
VAPB	c.166C > T	p.P56S	het	–	0.63	rs74315431	#3	m	fALS	41	Nishimura et al., 2004; Aliaga et al., 2013	Disease causing	probably damaging	damaging	–	–
VAPB	c.390T > G	p.D130E	het	0.15	0.63	rs146459055	#22	f	sALS	67	Suzuki et al., 2009	Disease causing	benign	tolerated	–	–
VAPB	c.479_481 delCTT	p.S160del	het	0.28	0.63	rs566283411	#677	f	sALS	72	Landers et al., 2008	Disease causing	–	–	–	–
VCP	c.1194+3G > A	p.?	het	0.05	0.63	rs183223259	#20	f	ALS-FTD	70	–	Disease causing	–	–	no effect	effect
ALS candidate genes, modifiers, risk factors																
ATXN1	c.1117C > T	p.R373C	het	<0.01	0.63	–	#34	f	ALS-FTD	54	–	Disease causing	Probably damaging	Damaging	–	–
ATXN1	c.511C > A	p.R171S	het	0	0.63	–	#38	f	ALS-FTD	70	–	Disease causing	Probably damaging	Damaging	–	–
DPP6	c.746C > T	p.T249M	het	–	0.63	–	#428	m	sALS	64	–	Disease causing	Possibly damaging	Tolerated	–	–
FGGY	c.1221 + 2T > C	p.?	het	0.45	3.13	rs41287704	#5	m	sALS	58	Kenna et al., 2013	Disease causing	–	–	Effect	Effect
FGGY	c.1221 + 2T > C	p.?	het	0.45	3.13	rs41287704	#21	f	sALS	61	Kenna et al., 2013	Disease causing	–	–	Effect	Effect
FGGY	c.1221 + 2T > C	p.?	het	0.45	3.13	rs41287704	#732	m	sALS	61	Kenna et al., 2013	Disease causing	–	–	Effect	Effect
FGGY	c.1221 + 2T > C	p.?	het	0.45	3.13	rs41287704	#739	m	sALS	72	Kenna et al., 2013	Disease causing	–	–	Effect	Effect

(Continued)

TABLE 2 | Continued

Gene	cDNA	Protein	Zygosity	MAF_EA (%)	MAF (%) in this study	dbSNP	Pat-ID	Gender	Subtype	AAO (years)	Reference	MT	PolyPhen2	SIFT	NG2	NN
FGGY	c.1221 + 2T > C	p.?	het	0.45	3.13	rs41287704	#33	m	sALS	63	Kenna et al., 2013	Disease causing	–	–	Effect	Effect
FGGY	c.1435T > C	p.C479R	het	<0.01	0.63	–	#703	m	fALS	76	–	Disease causing	Probably damaging	Damaging	–	–
FGGY	c.979A > C	p.N327H	het	0.13	1.25	rs34026954	#28	f	ALS	67	Kenna et al., 2013	Disease causing	Probably damaging	Damaging	–	–
FGGY	c.979A > C	p.N327H	het	0.13	1.25	rs34026954	#38	f	ALS-FTD	70	Kenna et al., 2013	Disease causing	Probably damaging	Damaging	–	–
GLE1	c.398G > A	p.R133Q	het	<0.01	0.63	–	#37b	m	sALS	74	–	Disease causing	Benign	Tolerated	–	–
GRN	c.545C > T	p.T182M	het	0.03	0.63	rs63750479	#16	m	sALS	67	Guerreiro et al., 2010	Polymorphism	Benign	Tolerated	–	–
GRN	c.229G > A	p.V77I	het	0.01	0.63	rs148531161	#749	m	sALS	46	Yu et al., 2010	Polymorphism	Benign	Tolerated	–	–
GRN	c.361G > A	p.V121M	het	0	0.63	–	#28	f	ALS	67	–	Polymorphism	Benign	Damaging	–	–
GRN	c.970G > A	p.A324T	het	0.12	0.63	rs63750541	#36	m	flail arm	39	Sleegers et al., 2008; Kenna et al., 2013	Polymorphism	Benign	Tolerated	–	–
ITPR2	c.2831C > T	p.P944L	het	<0.01	0.63	rs377598368	#22	f	sALS	67	–	Disease causing	Benign	Tolerated	–	–
ITPR2	c.3485T > G	p.V1162G	het	0.15	0.63	rs61757114	#373*	f	sALS	72	Kenna et al., 2013	Disease causing	Benign	Tolerated	–	–
ITPR2	c.1834G > A	p.A612T	het	<0.01	0.63	rs199523133	#422	f	sALS	61	–	Disease causing	Possibly damaging	Tolerated	–	–
ITPR2	c.8002G > A	p.A2668T	het	0.21	0.63	rs61757116	#677	f	sALS	72	Kenna et al., 2013	Disease causing	Benign	Tolerated	–	–
ITPR2	c.3635C > T	p.A1212V	het	<0.01	0.63	rs368911384	#741	f	sALS	64	Kenna et al., 2013	Disease causing	Probably damaging	Tolerated	–	–
ITPR2	c.1447G > A	p.V483I	het	0	0.63	–	#29	f	sALS	73	–	Disease causing	Probably damaging	Tolerated	–	–
ITPR2	c.3539G > A	p.R1180Q	het	0.62	0.63	rs35862420	#36	m	flail arm	39	Kenna et al., 2013	Disease causing	Benign	Tolerated	–	–
KIFAP3	c.518-5T > A	p.?	het	–	0.63	–	#419	m	sALS	72	–	Polymorphism	–	–	Effect	No effect
KIFAP3	c.1301T > G	p.F434C	het	0.23	0.63	rs116755924	#52	m	sALS	45	–	Disease causing	Probably damaging	Damaging	–	–
NEFH	c.1235G > A	p.R412Q	het	0.01	0.63	–	#534	m	sALS	58	–	Disease causing	Possibly damaging	Damaging	–	–
PON3	c.217G > T	p.G73C	het	–	0.63	–	#47	m	sALS	78	–	Disease causing	Probably damaging	Damaging	–	–
SLC1A2	c.236C > G	p.A79G	het	0.04	0.63	rs377633002	#524	f	sALS	60	Meyer et al., 1998	Disease causing	Benign	Tolerated	–	–
UNC13A	c.3080C > T	p.P1027L	het	1.83	0.63	rs200328448	#10*	m	sALS	71	Koppers et al., 2013; Kenna et al., 2013	Disease causing	Possibly damaging	Damaging	–	–
UNC13A	c.182C > T	p.T61M	het	0.45	0.63	rs140141294	#26**	m	PLS	66	Koppers et al., 2013; Kenna et al., 2013	Disease causing	Possibly damaging	Tolerated	–	–
UNC13A	c.1073A > G	p.Y358C	het	–	0.63	–	#30	m	sALS	60	–	Polymorphism	Possibly damaging	Tolerated	–	–

(Continued)

TABLE 2 | Continued

Gene	cDNA	Protein	Zygosity	MAF_EA (%)	MAF (%) in this study	dbSNP	Pat-ID	Gender	Subtype	AAO (years)	Reference	MT	PolyPhen2	SIFT	NG2	NN
UTR variants																
APEX1	c.*2A > T	p.?	het	0.5	0.63	rs17112002	#47	m	sALS	78	–					
FUS	c.-37C > T	p.?	het	–	0.63	–	#422	f	sALS	61	–					
FUS	c.*41G > A	p.?	het	0.86	0.63	rs80301724	#741	f	sALS	64	Sproviero et al., 2012					
SOD1	c.-8A > C	p.?	het	–	0.63	–	#46	f	ALS-FTD	75	–					
VAPB	c.-33C > G	p.?	het	–	0.63	rs201547974	#676	f	sALS	51	–					
Synonymous variants																
ATXN2	c.2088C > T	p.(=)	het	–	0.63	–	#22	f	sALS	67	–					
DAO	c.723C > T	p.(=)	het	0.23	1.25	rs149956241	#25**	f	flail leg	54	–					
DAO	c.723C > T	p.(=)	het	0.23	1.25	rs149956241	#41	m	sALS	42	–					
DCTN1	c.3669T > C	p.(=)	het	0	0.63	–	#12	m	sALS	61	–					
DCTN1	c.3474A > G	p.(=)	het	–	0.63	–	#54	f	ALS-FTD	55	–					
DPP6	c.693T > C	p.(=)	het	–	0.63	–	#24*	m	sALS	70	Kenna et al., 2013					
FUS	c.1080C > T	p.(=)	het	0.05	0.63	rs190724342	#35	f	sALS	49	Kenna et al., 2013					
HEXA	c.1216C > T	p.(=)	het	0.02	0.63	rs140482769	#7	m	sALS	71	–					
HEXA	c.744C > T	p.(=)	het	–	0.63	–	#749	m	sALS	46	–					
ITPR2	c.4962G > A	p.(=)	het	0.69	0.63	rs191789657	#16	m	sALS	67	Kenna et al., 2013					
ITPR2	c.5569C > T	p.(=)	het	0.12	1.25	rs191281974	#24*	m	sALS	70	Kenna et al., 2013					
ITPR2	c.5569C > T	p.(=)	het	0.12	1.25	rs191281974	#40	f	sALS	43	Kenna et al., 2013					
ITPR2	c.6162C > T	p.(=)	het	<0.01	0.63	–	#31	f	sALS	47	–					
NEFH	c.2061A > G	p.(=)	het	–	0.63	–	#16	m	sALS	67	–					
NEFH	c.2646C > T	p.(=)	het	0.01	0.63	rs528790943	#422	f	sALS	61	Kenna et al., 2013					

(Continued)

TABLE 2 | Continued

Gene	cDNA	Protein	Zygosity	MAF_EA (%)	MAF (%) in this study	dbSNP	Pat-ID	Gender	Subtype	AAO (years)	Reference	MT	PolyPhen2	SIFT	NG2	NN
PON1	c.603G > A	p.(=)	het	0.17	0.63	rs148452713	#729**	m	sALS	40	Kenna et al., 2013					
SETX	c.6675C > T	p.(=)	het	<0.01	0.63	rs200382898	#33	m	sALS	63	-					
SLC1A2	c.846C > A	p.(=)	het	<0.01	0.63	rs376593061	#46	f	ALS-FTD	75	-					
SLC1A2	c.450G > A	p.(=)	het	0	0.63	-	#52	m	sALS	45	-					
SPG11	c.6258G > T	p.(=)	het	0.81	0.63	rs150761878	#13	m	sALS	73	Kenna et al., 2013					
UNC13A	c.771C > G	p.(=)	het	3.02	0.63	rs146739681	#3	m	fALS	41	Kenna et al., 2013					
UNC13A	c.4560C > T	p.(=)	het	0.1	0.63	rs141334897	#26**	m	PLS	66	-					
UNC13A	c.4143G > A	p.(=)	het	-	0.63	-	#26**	m	PLS	66	-					
UNC13A	c.2220G > A	p.(=)	het	0.17	0.63	rs201361019	#32	m	sALS	46	-					
VCP	c.832T > C	p.(=)	het	0.04	0.63	rs200670526	#625	m	fALS	53	-					

*Patients carry ≥30 C9orf72 repeat units. **Patients carry intermediate lenght C9orf72 repeats. *MAF*, minor allele frequency; *MAF_EA* is the maximum population frequency of the variant observed in the European American population in dbSNP, EVS or ExAC. *AAO*, age at onset; *MT*, MutationTaster (http://www.mutationtaster.org/); PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/); SIFT (http://provean.jcvi.org/genome_submit_2.php); NG2, NetGene2 (https://www.cbs.dtu.dk/services/NetGene2/); NN, Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html).

FIGURE 1 | Detection and filtering of variants.

modifiers included in our gene panel (**Figure 2**). In our example, each of the proteins interacts in the context of key proteins for motor neuron degeneration (except *CHCHD10* and *PON3*), pointing toward possible modifying effects of certain variants.

We searched our cohort for patients who carry multiple variants in ALS-associated genes and could identify 15 individuals carrying at least two variants (18.8%, synonymous variants excluded) in ALS-associated genes (**Table 4**). For example, missense variants in the *ITPR2* gene were found in co-occurrence with clearly or potentially pathogenic variants in seven (8.75%) individuals. Four of these variants were also detected in an ALS cohort screening by Kenna et al. (2013). In our cohort the mean age of onset in patients who carried a variant in the *ITPR2* gene in co-occurrence was 64.0 years compared to 66.6 years in patients carrying any other variants in co-occurrence (differences are not statistically significant, Wilcoxon–Mann–Whitney test). We detected two additional synonymous variants in *ITPR2* but according to current knowledge we cannot assess their actual impact on the ITPR2 protein. Four of the 15 patients carried an expanded (Pat #10 and Pat #373) or intermediate (Pat #26 and Pat #729) *C9orf72* repeat expansion in co-occurrence.

The mean age of onset in patients where no variant could be detected was 57.8 years, patients who carried one variant showed

A9

TABLE 3 | Carriers of pathogenic and intermediate *C9orf72* repeat expansions.

Pat-ID	Gender	Subtype	AAO (years)	*C9orf72*	n repeats	Additional variants
#2	m	sALS	49	Positive	30	
#10	m	sALS	71	Positive	35	*FIG4* c.1940A > G; p.Y647C (het.); *UNC13A* c.3080C > T; p.P1027L (het.)
#24	m	sALS	70	Positive	31	
#373	f	sALS	72	Positive	34	*ITPR2* c.3485T > G; p.V1162G (het.)
#673	f	sALS	76	Positive	n.a.	
#6	m	sALS	68	Intermediate	26	
#25	f	flail leg syndrome	54	Intermediate	27	
#26	m	sPLS	66	Intermediate	27	*UNC13A* c.182C > T; p.T61M (het.); *ALS2* c.1816-8C > T; p.?
#729	m	sALS	40	Intermediate	27	*SPG11* c.5381T > C; p.L1794P (het.)

a mean age of onset of 61.3 years and patients carrying two or more variants had a mean age of disease onset of 65.0 years. In comparison, the overall mean age of disease onset in our cohort was 60.1 years. However, these differences in age of onset are not statistically significant (Kruskal–Wallis Rank Sum Test).

Variants in Other NDD Genes

To match the hypothesis of common pathways in different neurodegenerative diseases (NDDs) and to link ALS to other entities of the NDD spectrum, we additionally searched for potentially pathogenic or disease causing variants in 238 genes which are associated with possible differential diagnoses or overlapping phenotypes that are included in our NDD gene panel. We identified 12 patients who carried potentially pathogenic variants in genes that are linked to other entities (**Table 5**).

In patient #38, we detected two heterozygous variants in the *GBE1* gene (p.S378R and p.P40T, see **Table 5**). Mutations in *GBE1* can cause autosomal recessively inherited adult Polyglucosan body disease (APBD) which is characterized by upper motor neuron signs similar to ALS, early neurogenic bladder, cognitive impairment and decreased or absent activity of the glycogen branching enzyme (Klein, 2013). APBD is one of the conditions that should be considered when establishing the diagnosis of ALS. Unfortunately, we could not investigate whether both variants occur in the compound-heterozygous state in our patient because samples for segregation analysis could not be obtained. Long-range PCR with mutation-specific primers was impossible due to the large distance of more then 170 kb between the variants.

Another interesting finding is the identification of heterozygous variants in the *SPG7* gene in four sporadic patients (see **Table 5**). Mutations in *SPG7* can cause autosomal recessively inherited spastic paraplegia type 7, but there are also some published cases of obviously autosomal dominant inheritance (e.g., Sánchez-Ferrero et al., 2013). The disease is mainly characterized by spasticity and weakness of the lower limbs. Additional neurologic symptoms might appear in more complex phenotypes. In our cohort, we identified the truncating mutation p.R213* and the missense mutations p.I743T and p.G349S which are both described as acting disadvantageous on SPG7 protein function (Brugman et al., 2008; Bonn et al., 2010). None of the four patients had further relevant variants in ALS associated genes (only one patient carries an additional missense variant of unknown clinical relevance in the *FGGY* gene).

We also identified a high number of variants in the *NOTCH3*, *SYNE1,* and *VPS13A* genes as expected in genes of this size. For *SYNE1*, as mainly loss-of-function mutations are considered as being pathogenic in motor neuron disease (Gros-Louis et al., 2007; Izumi et al., 2013; Noreau et al., 2013). Similarly, only variants which result in a loss or gain of one cysteine residue within epidermal growth factor (EGF)-like repeat domains (Dichgans et al., 2001) are considered pathogenic in *NOTCH3,* and for *VPS13A* mostly loss-of-function variants are considered as pathogenic (Tomiyasu et al., 2011). Thus we assume that detected variants in our cohort represent rare polymorphisms. We identified variants in further genes that are included in our gene panel (see **Table 5**) but are unlikely to be implicated in our patients' phenotypes.

By comparing the number of patients identified to carry potentially pathogenic variants in ALS related genes in our cohort with previously published cohort studies, we show that the frequency of affected genes may vary in different populations (**Table 6**). For example, in the *VAPB* gene we detected variants in 5% of German patients (four cases) whereas in other populations no variants in *VAPB* were identified at all. Striking differences in frequencies across populations can also be observed for *FIG4*, *FGGY*, *GRN*, *ITPR2,* and *UNC13A*. The studies used vastly different strategies for sequencing and variant evaluation and analyzed different gene sets [from 6 genes, partially hotspots only sequenced by Sanger in Chiò et al. (2012) to 169 genes sequenced by NGS in Couthouis et al. (2014)]. Thus we consider this comparison solely to hint toward possible differences in gene frequencies among populations as a consequence of founder effects.

DISCUSSION

By using next-generation sequencing we analyzed 39 ALS-associated genes in a German cohort of both familial and sporadic ALS patients. In total, we detected 54 rare variants in approved disease genes and possible candidate genes, risk factors, and modifiers (synonymous variants excluded) in 48 patients which represents 60% of our total cohort.

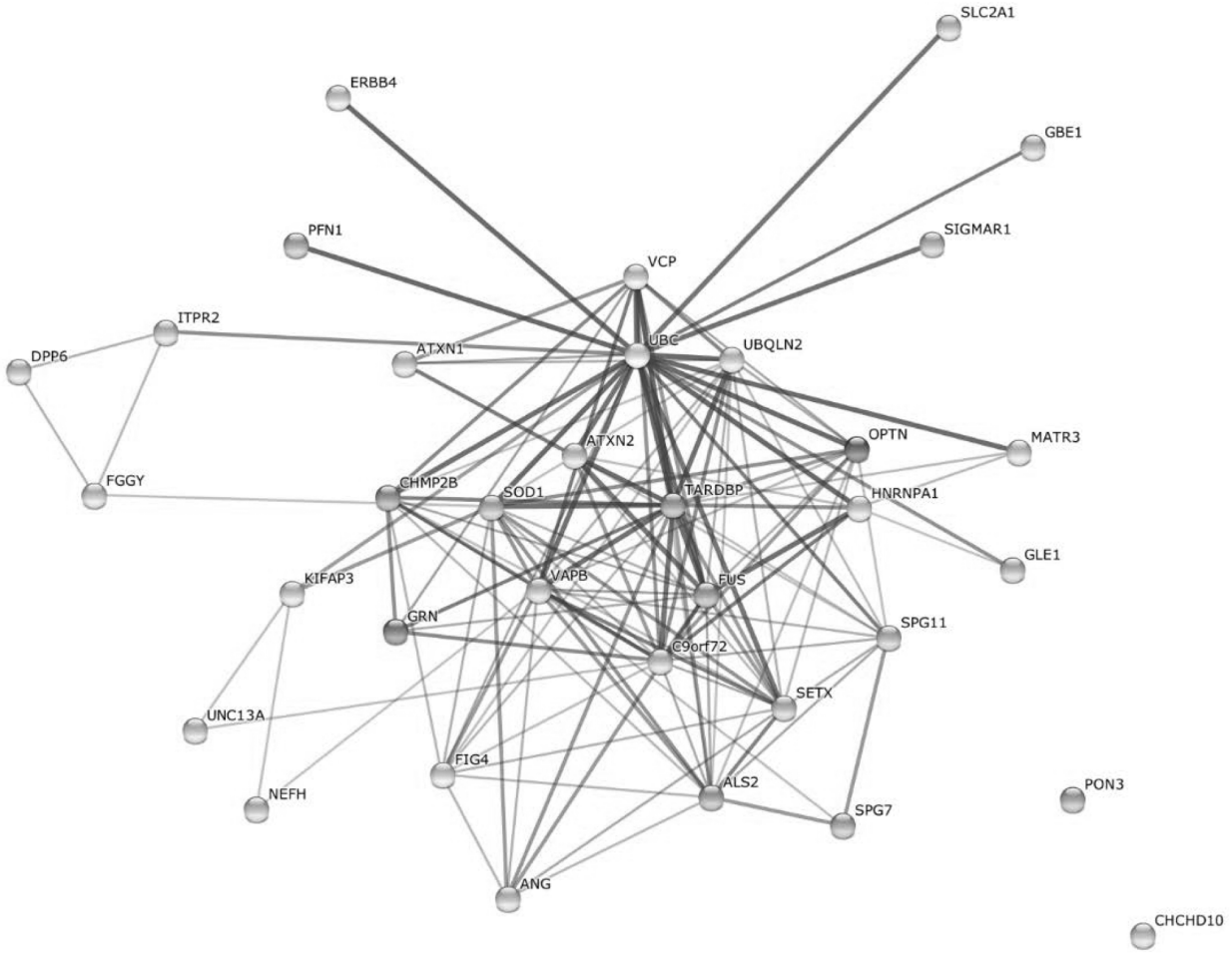

FIGURE 2 | Protein–protein-interaction network including 21 known ALS genes as well as 13 candidate genes, risk factors and possible modifiers included in our ALS gene panel (created with STRING10, Jensen et al., 2009). UBC, Ubiquitin C.

We identified pathogenic or potentially pathogenic variants in 14 analyzed disease genes in 20 patients of whom 19 patients (95%) are affected by sporadic ALS. This finding is unexpected, as it demonstrates that a genetic background can actually be found in a major proportion of seemingly sporadic cases (25%; 19 of 74 patients with sALS). We also would have expected to find more variants in familial cases. Although guidelines and recommendations on how to evaluate unknown variants are published (see for example Richards et al., 2015), the assessment of the actual pathogenicity of detected unknown variants with regard to the patients' phenotypes remains challenging and clear evidence on how a certain variant impairs the phenotype can only be achieved by extensive functional studies.

By focusing on possible candidate genes, risk factors, and modifiers, an interesting finding is the detection of heterozygous missense variants in the *GRN* gene in four patients affected by pure ALS (see **Table 2**). Three of the identified variants (p.V77I, p.V121M and p.A324T) are classified as being potentially pathogenic. Loss-of-function mutations in *GRN* are considered causative for frontotemporal lobar degeneration with ubiquitin-positive inclusions (Mackenzie et al., 2006). Recent evidence though suggests that missense mutations in *GRN* are also linked to the pathogenesis of ALS, especially as ALS and frontotemporal dysfunction are considered to represent a continuum of overlapping phenotypes, and a large proportion of ALS patients additionally experience frontotemporal dysfunction and vice versa (Sleegers et al., 2008; Cannon et al., 2013). Based on our findings, we recommend that *GRN* gene analysis should be included in routine molecular diagnostic settings and should also be considered in cases of pure ALS without frontotemporal involvement. Further, we detected seven missense variants in the *ITPR2* gene. Although Fernández-Santiago et al. (2011) as well as Chen et al. (2012) could not confirm an association of variants in *ITPR2* with ALS in a German and a Chinese cohort by SNP genotyping, we speculate that variation in the *ITPR2* gene could act as a modulating factor in ALS. A modulating effect might also exist for variants in *FGGY* (eight variants), *GRN* (four variants) and *UNC13A* (three variants).

These findings reflect the overall challenges in assessing the relevance of rare variants with respect to the phenotype as functional studies investigating the actual effect of these variants are largely missing. However, by the implementation of NGS in clinical genetics, we are now faced with increasing numbers of genes published as being possibly implicated in the pathogenesis of ALS. Such candidate genes gain further support from protein-protein interaction data. As rare variants

TABLE 4 | Co-occurrence of variants in ALS associated genes.

Pat-ID	Gender	Subtype	AAO (years)	Gene	cDNA	Protein	MAF_EA (%)	dbSNP
#10	m	sALS	71	*C9orf72*	Pathogenic repeat expansion			
				FIG4	c.1940A > G	p.Y647C	0.02	rs150301327
				UNC13A	c.3080C > T	p.P1027L	0.65	rs200328448
#22	f	sALS	67	*ATXN2*	c.2088C > T	p.(=)	-	-
				ITPR2	c.2831C > T	p.P944L	0.01	rs377598368
				VAPB	c.390T > G	p.D130E	0.1	rs146459055
#26	m	PLS	66	*C9orf72*	Intermediate repeat expansion			
				ALS2	c.1816-8C > T	p.?	0.38	rs185911369
				UNC13A	c.4560C > T	p.(=)	0.07	rs141334897
				UNC13A	c.4143G > A	p.(=)	-	-
				UNC13A	c.182C > T	p.T61M	0.2	rs140141294
#422	f	sALS	61	*ALS2*	c.4119A > G	p.I1373M	0.49	rs61757691
				FUS	c.-37C > T	p.?	-	-
				ITPR2	c.1834G > A	p.A612T	0.01	rs199523133
				NEFH	c.2646C > T	p.(=)	-	-
#524	f	sALS	60	*ALS2*	c.1816-8C > T	p.?	0.38	rs185911369
				SLC1A2	c.236C > G	p.A79G	0.01	rs377633002
#677	f	sALS	72	*ITPR2*	c.8002G > A	p.A2668T	0.29	rs61757116
				VAPB	c.479_481delCTT	p.S160del	0.45	rs566283411
#741	f	sALS	64	*FUS*	c.*41G > A	p.?	0.58	rs80301724
				ITPR2	c.3635C > T	p.A1212V	-	rs368911384
				TARDBP	c.931A > G	p.M311V	-	rs80356725
#29	f	sALS	73	*ITPR2*	c.1447G > A	p.V483I	-	-
				SETX	c.7358A > G	p.K2453R	-	-
#28	f	ALS	67	*FGGY*	c.979A > C	p.N327H	0.1	rs34026954
				GRN	c.361G > A	p.V121M	-	-
#34	f	ALS-FTD	54	*ATXN1*	c.1117C > T	p.R373C	-	-
				ATXN2	c.2049A > T	p.L683F	-	-
#36	m	flail arm	39	*GRN*	c.970G > A	p.A324T	0.14	rs63750541
				ITPR2	c.3539G > A	p.R1180Q	0.76	rs35862420
#38	f	ALS-FTD	70	*ATXN1*	c.511C > A	p.R171S	-	-
				FGGY	c.979A > C	p.N327H	0.1	rs34026954
#47	m	sALS	78	*APEX1*	c.*2A > T	p.?	0.66	rs17112002
				PON3	c.217G > T	p.G73C	-	-
#373	f	sALS	72	*C9orf72*	Pathogenic repeat expansion			
				ITPR2	c.3485T > G	p.V1162G	0.15	rs61757114
#729	m	sALS	40	*C9orf72*	Intermediate repeat expansion			
				PON1	c.603G > A	p.(=)	0.12	rs148452713
				SPG11	c.5381T > C	p.L1794P	0.01	rs201689565
#3	m	fALS	41	*UNC13A*	c.771C > G	p.(=)	0.85	rs146739681
				VAPB	c.166C > T	p.P56S	-	rs74315431
#16	m	sALS	67	*GRN*	c.545C > T	p.T182M	0.03	rs63750479
				ITPR2	c.4962G > A	p.(=)	0.36	rs191789657
				NEFH	c.2061A > G	p.(=)	-	-

(Continued)

TABLE 4 | Continued

Pat-ID	Gender	Subtype	AAO (years)	Gene	cDNA	Protein	MAF_EA (%)	dbSNP
#24	m	sALS	70	*C9orf72*	pathogenic repeat expansion			
				DPP6	c.693T > C	p.(=)	-	-
				ITPR2	c.5569C > T	p.(=)	0.17	rs191281974
#749	m	sALS	46	*GRN*	c.229G > A	p.V77I	0.01	rs148531161
				HEXA	c.744C > T	p.(=)	-	-
#33	m	sALS	63	*FGGY*	c.1221+2T > C	p.?	0.45	rs41287704
				SETX	c.6675C > T	p.(=)	0.01	rs200382898
#46	f	ALS-FTD	75	*SLC1A2*	c.846C > A	p.(=)	-	-
				SOD1	c.-8A > C	p.?	-	-
#52	m	sALS	45	*KIFAP3*	c.1301T > G	p.F434C	0.23	rs116755924
				SLC1A2	c.450G > A	p.(=)	-	-

in ALS associated genes according to current knowledge rather represent modifiers with effect on risk of developing the disease, age of onset, severity, or progression rate than disease causing mutations, further effort has to be made to understand how these modulating effects become evident in ALS. Investigating such modulating effects might lead to the identification of pathways that are not yet linked to ALS, enhancing our knowledge of ALS pathogenesis and higher-level neurodegenerative processes.

By performing repeat length analysis we identified five sporadic patients (6.25%) carrying pathogenic repeat expansions in the *C9orf72* gene. This is in line with Majounie et al. (2012) who reported on 5.2% of *C9orf72* repeat expansion carriers amongst German ALS patients. In two carriers of a pathogenic repeat expansion, we detected additional variants in ALS-associated genes. Although van Blitterswijk et al. (2012) suggested that additional genetic factors contribute to ALS pathogenesis in some carriers of a pathogenic *C9orf72* repeat expansion, we cannot assess the impact of additional variants on the patients' phenotypes in our cohort study. We identified four further patients carrying intermediate length repeat expansions. According to recent literature, these might be pathogenic in ALS as patients carrying 20–29 repeats are phenotypically similar to those with more than 30 repeats (Byrne et al., 2014). However, as intermediate length repeats have been detected in both patients and healthy controls, their actual pathogenicity still remains unclear (Rohrer et al., 2015). Of the four individuals with intermediate length repeat expansions, two patients carried additional variants in disease related genes. In our cohort, patients with intermediate length repeats had an earlier age of onset than carriers of a pathogenic repeat expansion (averages of 57.0 and 67.6 years, respectively). This counter-intuitive result leads us to speculate that age of onset was not primarily influenced by the length of repeat expansions but possibly by other factors such as additional variants in other genes. However, we cannot draw a firm conclusion due to our limited cohort size. Surprisingly, we did not detect pathogenic repeat expansions in any of the familial cases, although this might also be because of the small sample size.

To evaluate the hypothesis that ALS might be of complex genetic origin, we searched our cohort for patients carrying more than one potentially disease-causing variant. We found that 15 patients (18.8% of our cohort, synonymous variants excluded) carry two or more variants in ALS-associated genes and that four of these 15 patients additionally carry an expanded or intermediate *C9orf72* repeat expansion. According to current findings, a complex model of inheritance is used to explain phenomena like reduced penetrance or even intrafamilial phenotypic variability. A hypothesis by Cady et al. (2015) for example implies that disease onset is influenced by the burden of rare variants in ALS-associated genes. The authors reported that 3.8% of 391 study participants harbored two or more variants in 17 analyzed disease genes and that these individuals had disease onset 10 years earlier than patients carrying only one variant. The considerable difference in percentage of patients carrying two or more variants (3.8% in Cady et al., 2015 vs. 18.8% in this study) might be explained by the fact that we included not only variants in approved disease genes but also in candidate genes, modifiers, and risk factors. In contrast, Cirulli et al. (2015) did not report an effect of the number of variants on the age of onset in their cohort of 2869 ALS patients and 6405 controls, but they do not draw a strong conclusion as they did not test for pathogenic *C9orf72* repeat expansions. In our data, we do see a later age of onset in patients carrying two or more variants. However, due to our smaller sample size, we cannot make statistically significant observations on a possible correlation and we cannot exclude that co-occurrence of multiple variants might have a disadvantageous effect on disease onset, severity, disease duration, or site of onset by affecting disease causing variants. As an example, the identification of *ITPR2* variants in co-occurrence in seven patients might hint at a possible negative effect of additional variants in the *ITPR2* gene. Further studies should include both next-generation sequencing and tests for pathogenic repeat expansion in a large cohort to resolve this open question.

TABLE 5 | Detected variants in other NDD genes.

Pat-ID	Gender	Subtype	AAO (years)	Gene	chr: position	cDNA	Protein	Zygosity	MAF_EA (%)	dbSNP	Differential diagnoses (OMIM)	MT	PolyPhen2	SIFT	NG2	NN
#5	m	sALS	58	*AR*	chrX:66941751	c.2395C > G	p.Q799E	hemi	0.22	rs137852591	#300068, #312300, #300633, #313200	Disease causing	-	Damaging	-	-
#9	m	sALS	66	*GBA*	chr1:155209737	c.247C > T	p.R83C	het	0.01	rs1141812	#608013, #230800, #230900, #231000, #231005, #127750, #168600	Polymorphism	Probably damaging	Damaging	-	-
#18	m	sALS	42	*PLP1*	chrX:103043442	c.696+3G > A	p.?	hemi	-	-	#312920, #312080	Disease causing	-	-	No effect	No effect
#24*	m	sALS	70	*GARS*	chr7:30665930	c.1694T > A	p.L565Q	het	0.02	rs200726600	#601472, #600794	Disease causing	Benign	Damaging	-	-
#385	f	sALS	69	*GARS*	chr7:30655580	c.1100A > G	p.N367S	het	0.04	rs192443850		Disease causing	Benign	Tolerated	-	-
				SPG7	chr16:89592755	c.637C > T	p.R213*	het	-	-	#607259	Disease causing	-	-	-	-
#780	m	fALS	56	*DYNC1H1*	chr14:102452918	c.2356C > T	p.R786C	het	-	-	#614228, #614563, #158600	Disease causing	Probably damaging	Damaging	-	-
#34	f	ALS-FTD	54	*DYNC1H1*	chr14:102508609	c.12259G > A	p.A4087T	het	-	-		Disease causing	Probably damaging	Damaging	-	-
#38	f	ALS-FTD	70	*GBE1*	chr3:81640290	c.1134T > G	p.S378R	het	0.04	rs36099971	#263570, #232500	Disease causing	Benign	Tolerated	-	-
				GBE1	chr3:81810551	c.118C > A	p.P40T	het	0.17	rs35196441		Disease causing	Benign	Damaging	-	-
#41	m	sALS	42	*SPG7*	chr16:89623341	c.2228T > C	p.I743T	het	-	-	#607259	Disease causing	Probably damaging	Damaging	-	-
#19	m	sALS	54	*SPG7*	chr16:89598369	c.1045G > A	p.G349S	het	0.17	rs141659620		Disease causing	Probably damaging	Damaging	-	-
				SYNE1	chr6:152712567	c.7870C > T	p.R2624W	het	-	-	#612998, #610743	Polymorphism	Probably damaging	Damaging	-	-
#28	f	sALS	67	*SPG7*	chr16:89598369	c.1045G > A	p.G349S	het	0.17	rs141659620	#607259	Disease causing	Probably damaging	Damaging	-	-
#32	m	sALS	46	*TAF1*	chrX:70680560	c.5366A > G	p.N1789S	hemi	0.03	rs147517498	#314250	Disease causing	Benign	Damaging	-	-

**Patient carries a pathogenic C9orf72 repeat expansion.*

To genetically and mechanistically link ALS to other pathologies of the NDD spectrum, we searched our cohort for potentially pathogenic variants in 238 genes that are associated with overlapping phenotypes and are covered by our diagnostic panel.

We identified potentially pathogenic variants in neurodegeneration-related genes in 12 patients. Although compound-heterozygosity for the detected variants in *GBE1* in pat #38 is not proven, we speculate that both variants might be at least concurrently causative, especially as the patient revealed UMN-dominant ALS, cognitive impairment, and progressive non-fluent aphasia (PNFA) upon his last clinical examination in 2012. GBE1 is a glycogen branching enzyme which is involved in glycogen synthesis. According to Ngo and Steyn (2015), there is a link between the selective degeneration of neurons in ALS and metabolic alterations: Deficits caused by decreased glucose metabolism may trigger hyperexcitability and subsequent selective degeneration of upper and lower motor neurons. Although the underlying mechanisms are still unclear, Wang et al. (2015) could show that the FUS protein (juvenile ALS) interacts to a great extent with mitochondrial enzymes and proteins involved in glucose metabolism. With regard to these presumptions, we speculate that pathogenic variants in *GBE1* might be causative

TABLE 6 | Percentage of patients carrying potentially pathogenic variants in ALS associated genes (missense, splicing, small Indels only) (American: Couthouis et al., 2014; Irish: Kenna et al., 2013; Italian: Chiò et al., 2012; British: Morgan et al., 2015).

Gene	Our cohort (%) $n = 80$	American (%) $n = 242$	Irish (%) $n = 444$	Italian (%) $n = 475$	British (%) $n = 95$
SOD1	1.25	1.65	0	2.1	2.11
ALS2	5	1.24	1.35	-	5.26
SETX	2.5	2.07	2.25	–	–
SPG11	3.75	4.13	1.58	–	17.89
FUS	2.5	0.41	0.45	0.21	1.05
VAPB	5	0	0	–	–
ANG	0	0.41	0	0	–
TARDBP	1.25	–	0.45	1.47	2.11
FIG4	2.5	0.83	0	–	–
OPTN	0	0	0.23	0.21	2.11
ATXN2	1.25	1.22	0	–	–
VCP	1.25	0	0.23	–	–
CHMP2B	0	0	0.45	–	–
C9orf72-Repeat	6.25	1.65	8.78	6.74	–
APEX1	1.25	–	–	–	–
ATXN1	2.5	–	–	–	–
CCS	0	–	–	–	–
DAO	0	–	–	–	–
DCTN1	0	2.07	0.45	–	–
DPP6	1.25	1.65	0.23	–	–
FGGY	10	0.41	0.23	–	–
GLE1	1.25	–	–	–	–
GRN	5	0.41	0	–	–
HEXA	0	–	–	–	–
HFE	0	1.65	0.23	–	–
ITPR2	8.75	1.24	0.23	–	–
KIFAP3	2.5	–	–	–	–
LIF	0	–	–	–	–
NAIP	0	–	–	–	–
NEFH	1.25	0.41	0	–	–
PON1	0	0	0	–	1.05
PON2	0	0	0.23	–	1.05
PON3	1.25	0	0	–	–
RNF19A	0	–	–	–	–
SLC1A2	1.25	–	–	–	–
SPAST	0	–	–	–	–
UNC13A	3.75	1.24	0.23	–	–
VEGFA	0	–	–	–	1.05
VPS54	0	–	–	–	–

A9

for ALS or motor neuron degeneration, and that metabolic processes and involved genes must be taken into account in ALS genetics.

We detected known heterozygous variants in *SPG7* (paraplegin) in four patients. Recent evidence suggests that mutations in *SPG7* might be relevant in PLS as Mitsumoto et al. (2015) reported on the identification of a pathogenic heterozygous variant in *SPG7* in a patient affected by PLS. Paraplegin is part of the metalloprotease AAA complex, an ATP-dependent proteolytic complex located on the inner mitochondrial membranes, and functions in controlling protein quality and ribosomal assembly. Ferreirinha et al. (2004) showed that paraplegin-deficient mice develop axonal swellings as a consequence of accumulation of mitochondria and neurofilaments in the spinal cord which precedes axonal degeneration by impaired anterograde axonal transport. Although further studies are needed to assess the functional role of *SPG7* in human motor neurons, these findings hint at an important role of *SPG7* in motor neuron survival and support our hypothesis, that paraplegin is implicated in the pathogenesis of ALS and those pathogenic mutations in *SPG7* must be taken into account regarding genetic testing in ALS.

In summary, our results support recent observations whereby a genetic background is implicated in the sporadic form of ALS to a higher extent than assumed so far, and strengthen the upcoming hypothesis of ALS being a distinct manifestation of higher-level neurodegenerative processes rather than representing a discrete entity. Further, our results contribute to current discussions on a possible pathogenicity of intermediate repeat expansion in the *C9orf72* gene, especially in the interplay with additional variants in other ALS associated genes. In contrast to previously published studies, we could not prove an earlier age of disease onset in patients carrying multiple variants but speculate that variants in the *ITPR2* gene might act as a modulating factor in ALS. Additionally, our results lead us to assume that variants in *GRN* and *SPG7* might be implicated in the pathogenesis of ALS which is in line with the aforementioned hypothesis of common neurodegenerative processes leading to distinct phenotypes. Surprisingly, we did not detect clearly pathogenic variants in *SOD1* in our cohort, even though this gene is supposed to have a high impact on disease, encouraging us to launch a debate on the actual significance of *SOD1* in Germany.

CONCLUSION

We investigated 39 ALS-associated genes in a German cohort of 80 familial and sporadic ALS patients utilizing next-generation sequencing. We identified 22 variants in disease-causing genes in 20 patients and additionally 32 variants in candidate genes, risk factors, and modifiers in 28 patients. Thus we detected variants in ALS-associated genes in 60% of our study participants, of whom the vast majority are sporadic cases. Surprisingly, pathogenic repeat expansions in *C9orf72* and potentially pathogenic variants in *SOD1* were both detected at lower frequencies than expected. Instead we identified potentially pathogenic variants in the *GRN* gene in four patients, indicating that the impact of *GRN* mutations is not limited to ALS-FTD and might account for pure ALS, too.

Furthermore, our cohort enabled us to evaluate the hypotheses that ALS is of complex genetic origin. According to this hypothesis, numerous variants have some degree of influence on the clinical phenotype caused by the pathogenic mutation. We did in fact identify patients carrying variants in more than one ALS-associated gene. In contrast to other studies, however, our results do not show that patients with multiple variants have an earlier age of onset.

As ALS should be seen in the context of wider neurodegenerative disorders, we investigated our cohort for potentially pathogenic variants in 238 neurodegeneration related genes. The most interesting findings are the identification of two variants in the *GBE1* gene that might be causative in a patient with UMN-dominant ALS and the detection of heterozygous variants in *SPG7* in four ALS patients. These findings would benefit from extensive high-throughput sequencing in large patient and control cohorts of different ethnic background in order to more accurately assess the overall variability in ALS-associated genes and to better evaluate their impact on the disease.

Our results support the notion that next-generation sequencing could help uncover the genetic heterogeneous basis of ALS and thus argue for the broader application of NGS techniques in routine diagnostic settings. Therefore, our results are of immediate relevance for clinical genetics as we recommend that genetic testing in German patients should be offered not only to those with familial ALS but also to those with apparently sporadic ALS. We propose a two-stage strategy starting with a *C9orf72* repeat analysis, followed by comprehensive gene panel sequencing if *C9orf72* negative. To meet the high number of possible differential diagnoses that mimic ALS, genes causing FTD, HSP, spinal muscular atrophy (SMA) and other entities that impair motor neuron function should be included. Whereas Sanger sequencing focused on a few commonly affected genes such as *SOD1*, panel sequencing offers the opportunity to cover all disease-associated genes in only one approach and thus reveals the genetic heterogeneity of ALS and increases detection rates. Additionally, panel sequencing allows for the detection of multiple variants acting on the individual phenotype which might enable statements for example on disease progression or severity. We hope that our results will contribute to deeper knowledge which will allow the identification of new therapeutic targets for example by interfering with distinct pathways or personalized therapeutic approaches in the future.

It was our aim to broaden the genetic landscape of ALS. We detected previously identified ALS-causing mutations, novel variants within recognized disease-causing genes and candidate genes, in addition to modifiers and risk factors. Assessing the impact of newly detected variants and their potential contribution to the ALS phenotype requires further investigation in order to determine their functional relevance. For several

patients who gave their informed consent, we collected fibroblasts to provide the basis for the necessary functional work up.

AUTHOR CONTRIBUTIONS

Study concept and design: SK, MS, JP, and SB. Acquisition of clinical data and blood sample collection: JP and TGr. Analysis and interpretation of genetic data: SK, FB, AS, and MM. Drafting of manuscript: SK. Critical revision of manuscript: SK, FB, AS, MM, MS, LS, TGa, TGr, JP, and SB.

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SUPPLEMENTARY MATERIAL

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for submission as a LETTER to the Journal of Neurology

A case of beta-propeller protein-associated neurodegeneration due to a heterozygous deletion of *WDR45*

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Andreas Hermann: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript drafting.

Hagen Kitzler: Collection and/or assembly of data, critical revision of manuscript.

Claudia Funke: Collection and/or assembly of data, critical revision of manuscript.

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Caterina Terrile: Collection and/or assembly of data, critical revision of manuscript.

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Andreas Hermann: no conflict of interest.

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Abstract

WDR45 mutations have been recently reported as a cause of static encephalopathy of childhood with neurodegeneration in adulthood (SENDA), a phenotypically distinctive subtype of X-linked neurodegeneration with brain iron accumulation (NBIA)[4, 6]. *WDR45* encodes a beta-propeller scaffold protein with a putative role in autophagy. Therefore the disease was renamed beta-propeller protein-associated neurodegeneration (BPAN)[4, 6]. We here report on a female patient suffering from a classical BPAN phenotype due to a novel heterozygous deletion of *WDR45*. An initial Sanger sequencing approach failed to uncover the molecular defect. Based on the typical clinical and neuroimaging phenotype a quantitative PCR (qPCR) of the *WDR45* coding regions was initiated showing a reduction of the gene dosage by 50 % compared to controls.

Keywords

Static encephalopathy of childhood with neurodegeneration in adulthood, SENDA, BPAN, beta-propeller protein-associated neurodegeneration, NBIA, WDR45

Case report

We here report a 30-year-old female patient born at term as the first child to healthy unrelated parents from Germany. While her early postnatal adaption was reported normal her psychomotor development was delayed with unaided sitting at age 12 month and assisted walking at age 2 years. She spoke first words at age 2 years and her expressive language remained limited to single words. She suffered from febrile convulsions from the age of 2.5 years on, followed by epilepsy diagnosed and treated by the age of three years. At that time she lost her language abilities and became incontinent. Her condition subsequently remained stable until the age of 24 years. She attended a school for handicapped and walked with assistance although her gait was spastic and ataxic. By the age of 24 years, she developed new signs including yelling, progressive gait disturbance, and swallowing deficits with the need of tube feeding by the age of 28 years. Since the age of 29 she was completely wheelchair-dependent. Morbus Wilson and Rett Syndrome had been excluded, no signs of retinitis pigmentosa were found. Neuroimaging results (Figure 1) revealed hypointesities within the basal ganglia suggestive of neurodegeneration with brain iron accumulation[2, 4, 7]. Sequencing of *PKAN* was negative.

At the age of 30 years she was unable to communicate or follow commands. She only had an intermittent fixation, showed a vertical gaze palsy, tetraspasticity without voluntary movements, bilateral club feet and spontaneously positive Babinski sign.

Genetics

Using Sanger sequencing we did not observe potentially pathogenic sequence variants in *WDR45* (data not shown)[3, 6]. We then broadened the genetic diagnostics including other atypical forms of NBIA (including *PLA2G6, C9ORF12, FTL, FA2H, ATP13A2, CP*) [2], and Nieman-Pick's disease due to the vertical gaze palsy (*NPC1, NPC2*). All of them gave negative results.

Due to the distinct classical clinical presentation and neuroimaging findings (**Figure 1**) we further analysed the *WDR45* gene by qPCR. By doing so we detected a heterozygous deletion of the entire *WDR45* gene (**Figure 1B**). Testing of parental blood-derived DNA suggested that the variant occurred *de novo*. The karyotype was normal.

Methylation of one copy of the X chromosome in each female cell may result in one cell population expressing the wild-type allele and the other expressing the mutant allele. Skewing of X-inactivation has been discussed as a modifying disease mechanism in BPAN providing a possible explanation for the strikingly uniform clinical presentation of males and females[1, 3]. While keeping in mind that methylation patterns observed in blood cells do not necessarily reflect those in the affected tissue, a skewed X-inactivation has been observed in 13 out of 15 patients analyzed likely resulting in the expression of the mutant allele[3]. In our patient, X-inactivation studies using the HUMARA assay indicated an extremely skewed methylation pattern (95:5) in genomic DNA derived from peripheral blood cells.

Conclusion

We report on a female with a characteristic BPAN phenotype caused by a heterozygous *WDR45* deletion. While this change has been initially missed in routine genetic testing it was subsequently identified in an extended screening strategy including qPCR. This analysis was initiated based on the distinct clinical features and course of the disease. CNVs affecting *WDR45* might therefore represent an underdiagnosed cause of neurodegenerative disorders. We suggest that an extended search for deletions should be performed in apparently *WDR45*-negative cases presenting with features of neurodegeneration with brain iron accumulation and might as well be considered in young patients with predominant intellectual disabilities and parkinsonism[5].

Methods

Sanger Sequencing

The coding region and the flanking exon-intron boundaries of the genes *ATP13A2, C19ORF12, CP, FA2H, FTL, NPC1, NPC2, PLA2G6* and *WDR45* were amplified by PCR method and screened by direct bidirectional Sanger sequencing according to standard protocols using the 3730xl DNA Analyzer (Applied Biosystems).

MLPA

Deletion and duplication analysis of the genes *PLA2G6, NPC1* and *NPC2* was performed by usage of Multiplex Ligation-dependent Probe Amplification (MLPA, MRC Holland, SALSA MLPA kits P120-B1 (*PLA2G6*) and P193-A2 (*NPC1, NPC2*)) according to the manufacturer´s protocol.

Quantitative real-time PCR

Deletions and duplications affecting the *WDR45* gene (NM_007075.3) were analyzed by quantitative real time PCR using the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) and the QuantStudio 12K Flex system (Life Technologies) according to the manufacturer´s protocol with intragenic amplicons in the coding exons 3 – 8, 10 and 12 and in three reference amplicons.

Humara assay

We used genomic DNA derived from whole blood cells to investigate X-inactivation patternsin the patient as described previously [1].

References:

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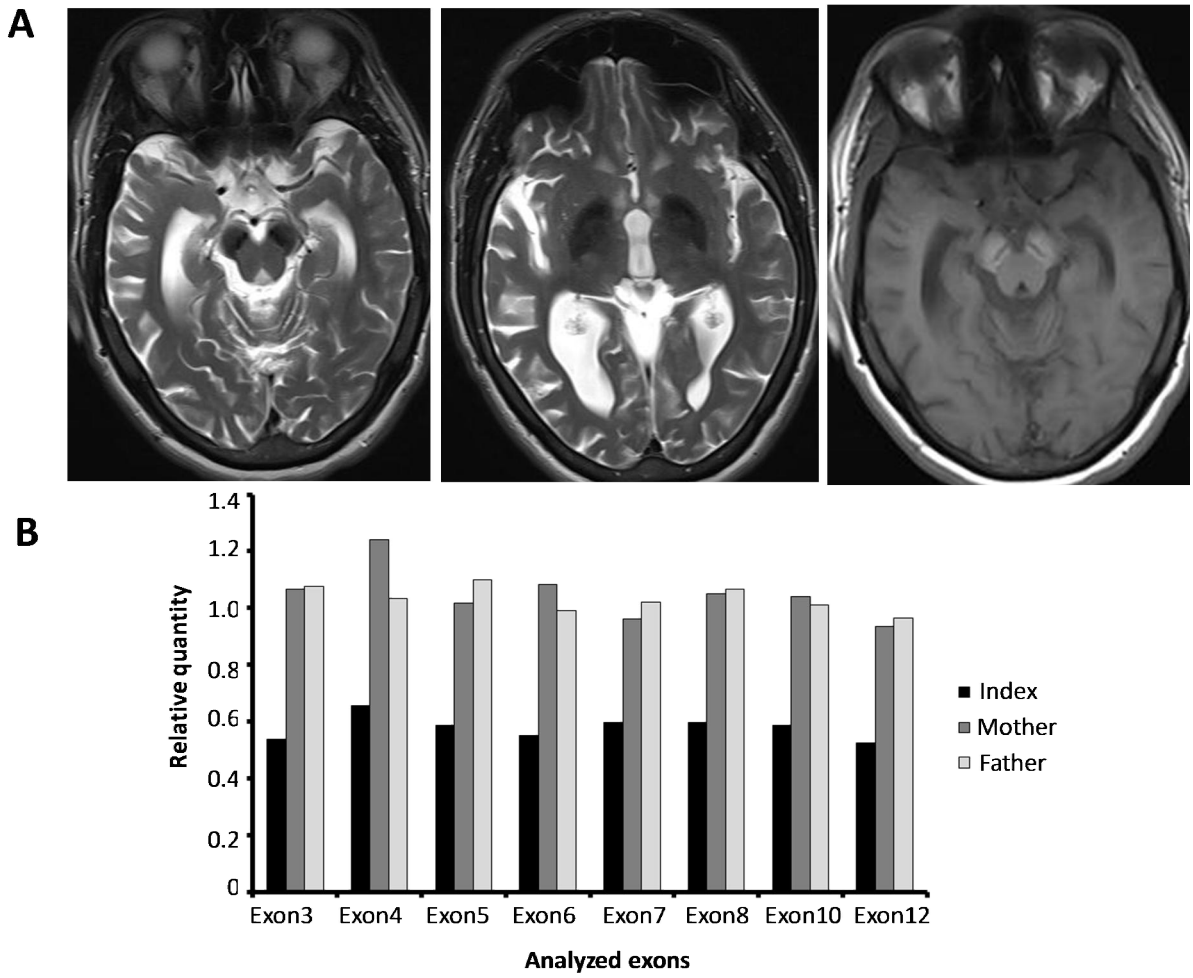
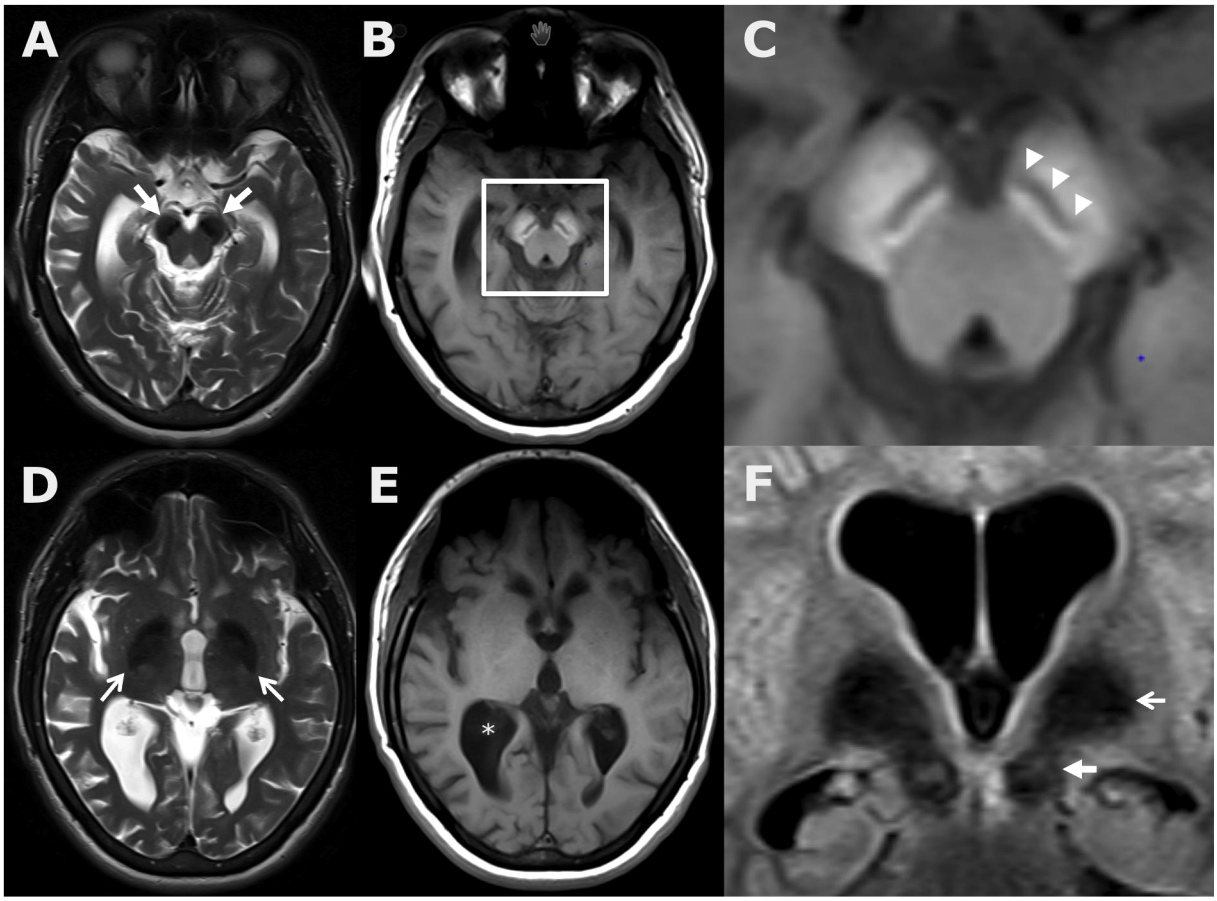


Neuroimaging and genetics of referred patient.

(A) T2-weigthed axial MRI images showing hypointense signals in the substatnia nigra and putamen suggestive of iron deposition. T1-weighted images depict the pathognomonic hyperintense signals around the substantia nigra.

(B) qPCR of the *WDR45* coding regions of the index patient and her parents showing a reduction of the gene dosage to approximately 50 % of controls suggestive of a heterozygous deletion of the entire *WDR45* gene.



Patient brain MRI panel displaying T2 weighted (*left column*) and T1 weighted scans of consecutive axial slices at mesencephalic and basal ganglia level: BPAN typical signal changes were found as symmetric cerebral peduncle including substantia nigra T2 hypointensity (A,F; *filled arrow*) combined with T1 hyperintensity (B) and a therein located circumscribed hypointense band (B and enlarged section C; *arrowheads*). This spatial signal characteristic is considered to be a nearly pathognomonic magnetic resonance imaging feature of BPAN. In contrast the further found symmetric T2 hypointensity of the globus pallidus (D,F; *open arrows*) was not accompanied by T1 signal changes (E). Secondarily general enlargement of the lateral ventricles (*) and cortical sulci suggested early cerebral parenchymal volume loss.