Novel bi-allelic variants expand the SPTBN4-related genetic and phenotypic spectrum

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Abstract
Neurodevelopmental disorder with hypotonia, neuropathy, and deafness (NEDHND, OMIM #617519) is an autosomal recessive disease caused by homozygous or compound heterozygous variants in SPTBN4 coding for type 4 βIV-spectrin, a non-erythrocytic member of the β-spectrin family. Variants in SPTBN4 disrupt the cytoskeletal machinery that controls proper localization of ion channels and the function of axonal domains, thereby generating severe neurological dysfunction. We set out to analyze the genetic causes and describe the clinical spectrum of suspected cases of NEDHND. Variant screening was done by whole exome sequencing; clinical phenotypes were described according to the human phenotype ontology, and histochemical analysis was performed with disease-specific antibodies. We report four families with five patients harboring novel homozygous and compound heterozygous SPTBN4 variants, amongst them a multi-exon deletion of SPTBN4. All patients presented with the key features of NEDHND; severe muscular hypotonia, dysphagia, absent speech, gross motor, and mental retardation. Additional symptoms comprised horizontal nystagmus, epileptiform discharges in EEG without manifest seizures, and choreoathetosis. Muscle histology revealed both characteristics of myopathy and of neuropathy. This report expands the SPTBN4 variant spectrum, highlights the spectrum of morphological phenotypes of NEDHND-patients, and reveals clinical similarities between the NEDHND, non-5q SMA, and congenital myopathies.

Introduction
Spectrins are cytoskeletal proteins found in a variety of tissues and cell types. They were initially identified in erythrocytes [1]. Vertebrate spectrin is a hetero-tetramer formed by two α- and two β-subunits. The α-spectrin sub-group has two members (I and II), while the β-spectrin subgroup has five members (I–V). βIV-spectrin is enriched in the myelinated neurons of the central nervous system [2], where it has a role in the clustering of sodium and potassium channels at the axon initial segment (AIS) and at the nodes of Ranvier via interaction with ankyrin-G [3]. The SPTBN4 gene encodes a non-erythrocytic βIV-spectrin. We published the first case of autosomal recessive myopathy caused by a homozygous pathogenic SPTBN4 variant in 2017 [4]. The proband was boy of Kurdish descent who, in addition to the classical symptoms of myopathy, exhibited sensorineural hearing loss, intellectual disability, and Spectrin-associated neuropathy. A recent report by Wang et al. [5] described six additional children with this disease due to autosomal recessive SPTBN4 variants. They demonstrated that a loss-of-function variant disrupted

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sodium and potassium channel clustering, leading to neuropathy. Häusler et al. reported a novel homozygous splice-site variant in a pair of siblings presenting with axonal neuropathy in the absence of intellectual disability [6]. In pigs, deletions in SPTBN4 cause severe myopathy [7]. We now report five additional affected individuals from four families who were found to harbor variants that affect function of SPTBN4. Our findings further define the clinical spectrum of neurodevelopmental disorder with hypotonia, neuropathy, and deafness.

Materials and methods

Patients

The patients’ parents provided written informed consent for study participation, including publication of patient photographs, in accordance with the Declaration of Helsinki (Charité Ethics Committee approval EA2/107/14). Patients 1 and 2 were admitted to the Helios Klinikum Hohenzülicken in Berlin, Germany, for neuro-rehabilitation; Patient 3 presented as an outpatient at UNC Chapel Hill’s Division of Pediatric Genetics and Metabolism, Chapel Hill, NC, USA; Patient 4 presented as an outpatient at the Iranian University of Medical Sciences, Tehran, Iran; and Patient 5 was treated and diagnosed at the Clínica Las Condes, Pediatric Neurology, Santiago, Chile.

Sequencing and segregation analysis

Genomic DNA was extracted from white blood cells and whole exome sequencing (WES) was performed for all patients. Variant interpretation was performed according to current ACMG guidelines for variant classification [8]. We submitted our variants to ClinVar (VCV000987745, VCV0009877446, VCV000987747, VCV000988586).

Histology, immunohistochemistry (IHC), and morphometry

Fiber-type-specific atrophy was measured by determining the minimal Feret’s diameter of each fiber from muscle cross-sections using a standardized method. The use of the minimal Feret’s diameter minimizes the confounding factor of oblique sectioning. For calculating the minimal Feret’s diameter all fibers of one representative 200× field of view of a ATPase pH 4.3 stained muscle section were counted. This amounted to n = 131 type 1 and n = 156 type 2 fibers in the patient, and n = 177 type 1 and n = 174 type 2 fibers in the control. IHC staining and imaging was performed as previously described [4]. Images were loaded into ImageJ (https://imagej.nih.gov/ij/) processing software, the circumference of individual fibers was manually traced and the minimal Feret’s diameter calculated using the build-in measuring algorithm. Values were visualized as cumulative histograms in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

Results

Clinical reports

Family A (Patients 1 and 2)

Two affected sisters were born at term to healthy first-cousin parents from Saudi Arabia. The pregnancy with Patient 1 was complicated by gestational diabetes. Muscular hypotonia was noted at birth. Feeding problems became apparent during the newborn period. She suffered from recurrent aspiration pneumonia and dysphagia from the first month of life, requiring gavage feeding from 1 year of age. Motor development was severely delayed, and the patient did not achieve early developmental milestones such as head control, rolling over, and crawling (Table 1). A physical examination at age 3 years revealed a high palate, myopathic facies, severe distal muscle weakness, generalized atrophy, bilateral ankle flexion contractures (Fig. 1A, B), and severe global developmental delay. Patient 1 was unable to stand, sit, eat, or drink without support at age 3 years. Her speech was limited to repeating single words in Arabic and German. Neurometabolic screening tests (tandem mass spectrometry of amino and organic acids, lactate, ammonia) and first-line genetic analyses (karyotyping, muscular dystrophy gene panel) were inconclusive. Serum CPK levels were normal. Cranial MRI revealed a diffuse T2-hyperintensity, predominantly affecting the subcortical white matter. Structural abnormalities were ruled out. MR spectroscopy was normal. A muscle biopsy was not performed. The younger sister of the patient (Patient 2) presented in a similar way but was more severely affected at the age of 2 years.

Family B (Patient 3)

Patient 3 is a 5-year-old female and the first child born to healthy paternal second cousins from Afghanistan. Pregnancy and birth were normal. Muscle hypotonia was diagnosed in the neonatal period. She suffered from recurrent pulmonary aspiration starting at 5–6 months of age. She had developed neither head control, independent sitting, crawling, nor talking by 2 years of age. A physical exam revealed horizontal nystagmus, choreoathetosis of the arms with intermittent dystonia, and generalized hypotonia (Fig. 1D). Deep tendon reflexes were brisk without clonus. Laboratory testing for inborn errors of metabolism, routine karyotyping, and...
Table 1 Genetic and clinical features of our patients with *SPTBN4* variants. Symptoms are encoded according to Human Phenotype Ontology (HPO) [14].

| Characteristics and symptoms | HPO | Pat 1, Fam A (this report) | Pat 2, Fam A (this report) | Pat 3, Fam B (this report) | Pat 4, Fam C (this report) | Pat 5, Fam D (this report) |
|-----------------------------|-----|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Mutation in *SPTBN4* (NM_020971) | c.3375_3393del p. (Asp1126Thrfs*39) | c.3375_3393del p. (Asp1126Thrfs*39) | c.737G>C p. (Arg246Pro) | c.1247del p. (Leu417Thrfs*5) | c.1149dup p. (Asn384Glnfs*17) / g. (?_41,001,394)_(41,011,375_?)del |
| ACMG variant classification | PVS1 | PVS1 | PM2 | PVS1 | PVS1 |
| Ethnicity | Saudi Arabia | Saudi Arabia | Afghanistan | Iran (Kurdish) | Chile (Latin American) |
| Gender | Female | Female | Female | Female | Female |
| Consanguinity | Y | Y | Y | Y | N |
| Zygosity | Hom | Hom | Hom | Hom | Comp Het |
| Head | Myopathic facies | HP:0002058 Y | Y | Y | Y | Y |
| Poor head control | Y | Y | Y | Y | Y |
| High palate | HP:0000218 Y | Y | Y | Y | N |
| Sensorineural hearing impairment | HP:0000407 N (clinically) | N (clinically) | N (clinically) | Y | N (clinically) | Y |
| Absent brainstem auditory responses | HP:0004463 ND | ND | Y | ND | N |
| Scoliosis | HP:0002650 N | N | N | Y | Y |
| Respiratory and chest | Recurrent infections due to aspiration | HP:0004891 Y | Y | Y | Y | Y |
| Gastrintestinal | Feeding difficulties | HP:0011968 Y | Y | Y | Y | Y |
| Poor suck | HP:0002033 Y | Y | Y | Y | Y |
| Dysphagia | HP:0002015 Y | Y | Y | Y | Y |
| Gastroesophageal reflux | HP:0002020 U | U | Y | Y | Y |
| Gastrostomy tube feeding in infancy | HP:0011471 Y | Y | Y | N | Y |
| Skeletal | Ankle contracture | HP:0006466 N | N | N | Y | Y |
| Neurologic | Neonatal hypotonia | HP:0001319 Y | Y | Y | Y | Y |
| Generalized hypotonia | HP:0001290 Y | Y | Y | Y | Y |
| Generalized muscle atrophy | HP:0009055 Y | Y | N | Y | Y |
| Distal limb muscle atrophy | HP:0003693 Y | Y | N | Y | Y |
| Choreoathetoid movements | HP:001266 N | N | Y | N | N |
| Abnormality of the cerebral white matter | HP:0002500 Y | ND | N | ND | N |
microarray analysis were inconclusive. Cranial MRI and CT were normal. MRS revealed nonspecific lipid and lactate peaks with increased glutamine/glutamate peaks in the region of the basal ganglia and the Corpus callosum. Oral feeding led to recurrent aspiration pneumonias and dystrophy with a body weight below the first percentile. Gastrostomy feeding was started at 3 years of age. Seventy-two-hour EEG showed abundant to nearly continuous centro-parietal sharp/spike/poly-spike wave discharges during sleep without clinical correlation to manifest seizures. Nerve conduction studies and EMG were entirely normal. Otoacoustic emissions were present. In brainstem evoked response audiometry (BERA) only wave 1 from the inner ear could be recorded pointing to a defect at the level of cochlear nerve conduction.

**Family C (Patient 4)**

Patient 4 is a 4-year-old female born to healthy consanguineous parents from Iran with Kurdish background. Her older brother died at age 2 years due to increasing feeding difficulties and subsequent aspiration pneumonia. His phenotype was described as similar. Patient 4 showed signs of general hypotonia and muscle weakness shortly after birth (Fig. 1F). Routine metabolic testing, including acylcarnitine and urine organic acids, was normal. Genetic testing for spinal muscular atrophy type 1 was negative. She had poor head control and neither sat, crawled, nor spoke, and had severe dystrophy (Fig. 1G). She presented with clinical signs of myopathy, including myopathic facies, high arched palate, and bilateral ankle flexion contractures. She had horizontal nystagmus. EEG or BERA were not recorded. Sensory-nerve action potentials and motor action potentials were normal.

**Family D (Patient 5)**

Patient 5 is a 7-year-old girl born to healthy nonconsanguineous parents from Chile. She was born at term, following a normal pregnancy and delivery. Her parents noted a weak suck and slow weight gain during her first week of life. Muscular hypotonia, motor delay, and frequent choking and gagging while feeding were noted at 3 months of age. Severe gastroesophageal reflux with nasal regurgitation led to recurrent respiratory infections. Patient 5 had attained only partial head control and could neither sit nor stand (Fig. 1I, J). An examination at 10 months of age showed mild weakness of facial musculature, severe hypotonia without trunk control, bilateral Talipes equinovarus and Pes cavus, and absent deep tendon reflexes. Though able to exert spontaneous antigravity movements of the upper and lower limbs, she was unable to lift her head when prone. Bilateral moderate to severe hearing loss was diagnosed at 18 months of age, at 3 years her speech recognition threshold was 80 dB HL. Her growth parameters at age 4

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**Table 1 (continued)**

| Characteristics and symptoms | HP0003448 | HP0003477 | HP0003554 | HP0003554 | HP0003554 |
|-----------------------------|-----------|-----------|-----------|-----------|-----------|
| Demyelinating peripheral neuropathy | N         | N         | N         | N         | N         |
| Peripheral axonal neuropathy | HP0011807 | HP0012044 | HP0012044 | HP0012044 | HP0012044 |
| Type 1 muscle fiber atrophy | N         | N         | N         | N         | N         |
| Type 2 muscle fiber atrophy | N         | N         | N         | N         | N         |
| Acroparesis | N         | N         | N         | N         | N         |
| Absent speech | N         | N         | N         | N         | N         |
| Premature birth (<37 weeks of gestation) | N         | N         | N         | N         | N         |
| Absent deep tendon reflexes | N         | N         | N         | N         | N         |
| Horizontal nystagmus | N         | N         | N         | N         | N         |
| EEG abnormality | N         | N         | N         | N         | N         |
| Premature manifestation | N         | N         | N         | N         | N         |
years were below the third percentile. Tracheostomy and gastrostomy were required due to increasing dysphagia and a weak cough reflex coupled with recurrent pneumonias. Karyotyping, serum CPK levels, Prader–Willi syndrome (tested by methylation analysis), and spinal muscular atrophy type 1 genetic testing were normal. cMRI did not show any abnormalities. Nerve conduction studies showed normal sensory and motor conduction velocities, with low-amplitude motor responses. EMG showed signs of acute and chronic denervation such as fibrillation potentials and positive sharp waves, motor unit potentials with increased amplitude and duration, and decreased recruitment. An EEG at age 6 years revealed almost continuous and severe generalized epileptiform activity without any clinical correlate of manifest seizures (Fig. 1L).

Whole exome sequencing identifies variants in SPTBN4

WES of the patients and their parents revealed novel bi-allelic variants in SPTBN4 in all four families. Deletions were found in Families A and C. Patient 1 and 2 from Family A were homozygous for [chr19:g.41,025,779_41,025,797del (hg19); NM_020971.3.c.3375_3393del; p.(Asp1126Thrfs*39)] (Fig. 1C) and Patient 4 from Family C was homozygous for [chr19:g.41,008,725_41,008,725del (GRCh37); NM_020971.3.c.1247del; p.(Leu417Tyrfs*5)]. These deletions cause frameshifts that lead to a premature stop codon. Sanger sequencing was done in Family A and verified the variant and its segregation. Both parents were heterozygous. Patient 3 from Family B was homozygous for the missense variant [chr19:g.41,003,464 (GRCh37); NM_020971.3.c.737G>C; p.(Arg246Pro)] (Fig. 1E) that was absent from gnomAD. Patient 5 from Family D was compound heterozygous for two variants. The paternally inherited single base insertion [chr19:g.41,008,360dup (GRCh37); NM_020971.3.c.1149dup; p.(Asn384Glnfs*17)] causes a frameshift that leads to a subsequent premature stop codon. The maternally inherited deletion with breakpoint spanning [chr19:g. (?_41,001,394)، 41,011,375_؟_del (GRCh37)] encompasses exons 6–11 of SPTBN4 (Fig. 1K).

Histology and morphometry

Muscle biopsy was performed in Patients 3 and 5. In Patient 3, ATPase pH 4.3 staining and IHC with an antibody
directed against myosin heavy chain slow (MHC neonatal, NCL-MHCn, Novocastra, 1:20) revealed a reduction of type 1 fiber diameters but no manifest fiber-type disproportion (Fig. 2A, B). Immunostaining of the same samples with an antibody directed against β-spectrin showed staining of the sarcolemma in both individuals (C, D, 600× and 200×, respectively). Staining for βIV-spectrin did not show any signal at the sarcolemmal position of the Patient 3 (F) in contrast to a healthy control (E) (400×). G Histogram of diameters from type 1 and type 2 muscle fibers from Patient 3 and an age-matched control showing an over-proportional thinning of type 1 fibers. H and I neurogenic changes with atrophic muscle fascicles and angulated fibers next to normally appearing muscle fibers in HE and Gomori-trichrome staining. J Summary of so far published and here described disease-causing variants in SPTBN4. NB, the multi-exon deletion is not marked on the graph.

For IHC we had cryopreserved muscle tissue only from Patient 3 at our disposal. IHC with an antibody directed against β-spectrin (NCL SPEC1, clone RB C2/3D5, Novocastra UK; 1:100) showed a strong sarcolemmal demarcation of muscle fibers in Patient 3 and an age-matched control (Fig. 2C, D). In contrast, IHC with an antibody directed against the non-erythrocytic βIV-spectrin subtype 4 (sc-368195, H-85, Santa-Cruz, 1:100) showed no staining in Patient 3, while staining was preserved in age-matched control muscle (Fig. 2E, F).
Discussion

Spectrins are molecular scaffold proteins that link the plasma membrane to the actin cytoskeleton. They are crucial for the determination of cell shape, arrangement of transmembrane proteins, and organelle organization. Variants in SPTBN4 result in ion-channel dysfunction by disrupting the cytoskeletal machinery that controls proper localization of channels and the function of axonal domains in the AIS and in the nodes of Ranvier, where axonal ion channels are normally clustered causing a variety of nervous system dysfunctions.

We have identified multiple novel variants that affect function of SPTBN4 in patients with severe muscular hypotonia, dysphagia, absent speech, delayed gross motor development, and intellectual disability. These symptoms are considered key features of the SPTBN4-related disorder. We have broadened the disorder’s clinical spectrum by describing the variably present features of areflexia, axonal motor neuropathy, nystagmus, epileptiform activity in EEG without clinical correlation, and a movement disorders with choreoathetosis.

Analysis of muscle biopsy specimens from affected infants revealed signs of a primary myopathy as well as of secondary neuropathic features. Electrophysiology revealed signs of obvious neuropathy only in Patient 5. This was in accordance with the neuropathic pattern characterized by neurogenic fiber-type grouping in the histopathological studies for this patient. βIV-spectrin was absent from the sarcolemma of Patient 3, while ATPase staining showed hypotrophic type 1 fibers, but no clear fiber-type disproportion, a finding more characteristic for congenital myopathies. We hypothesize that βIV-spectrin deficiency directly impacts the structural stability of the sarcolemma and the initiation or propagation of the depolarization waves along the myofiber and its T-tubular system. We derived that the evidence for a myopathy is mostly from the clinical and histopathological findings, but not from functional studies about the role of SPTBN4 in muscle cells. Further studies are thus needed to determine the impact of pathogenic SPTBN4 variants on the muscle cells proper. Our clinical phenotyping and neurophysiological studies suggest that the muscle weakness seen in patients with SPTBN4 disorder may be caused by a combination of axonal neuropathy and congenital myopathy. This does not rule out the possibility that one pathological principle may dominate as described by Wang et al. (2018), where neuropathy seemed to be the predominant feature.

Though two of our patients did not exhibit clinically manifest seizures, their EEGs showed highly pathologic epileptiform discharges. As βIV-spectrin plays a role in the clustering of KCNQ2 subunit-containing potassium channels, there could be some degree of overlap of clinical symptoms with early-infantile epileptic encephalopathy type 7 and with benign familial neonatal seizures type 1. This is supported by Wang et al. who reported that three of their six patients had clinically manifest epilepsy, with two patients being refractory to antiepileptic medication. As variants in KCNQ2 cause a wide range of phenotypes even within single families with members sharing the same variant [9], we can extrapolate that this may also apply to a protein involved in KCNQ2 clustering. A review of all reported cases of SPTBN4-related disorders shows that epileptic activity and seizures are more common than initially thought. In contrast to the pair of siblings described by Häusler et al. [6], all our patients with a molecular diagnosis of SPTBN4 disorder had severe intellectual disability, indicating that intellectual disability is frequent in this condition. This information must be taken into account when counseling patients and families with SPTBN4 disorder.

We identified and described five additional cases with pathogenic bi-allelic SPTBN4 variants. Four of these were novel and two resulted in a frameshift. The pathogenic homozygous missense variant identified in Patient 3 led the exchange of an evolutionary conserved proline for an arginine. This variant was absent in the gnomAD database and in ClinVar [10]. Patient 5 harbors a multi-exon deletion on the maternally inherited allele and a small insertion leading to a frameshift on the paternally inherited allele. This is the first description of a multi-exon deletion in the SPTBN4 gene and it shows that larger SPTBN4 deletions may account for a part of SPTBN4-related disorder. Screening for large genomic SPTBN4 rearrangements should improve molecular diagnostic rates for this population, in particular for patients where only a single variant that affects function has been identified.

To date, including our report, 15 pathogenic variants—truncating (n = 10), missense (n = 4), splice-site (n = 1) variants, and multi-exon deletion (n = 1)—have been reported in SPTBN4 [4–6, 11–13] (Fig. 2J). Most affected individuals reported to date are homozygous. In this report we present the first individual with a multi-exon deletion. Patients generally suffer from severe developmental delay and intellectual disability, although two individuals in one family had a milder phenotype, including one individual with normal cognitive development. Speech and language skills are often severely limited. A affected individuals rarely achieve head control and are unable to sit, stand, or walk. They typically have congenital muscular hypotonia. Axonal motor neuropathy leads to hyporeflexia/areflexia and weakness. Most affected individuals require tube feeding. More than half of them develop seizures or have a pathological EEG. The mutations are dispersed over the whole gene and we do not see a clear genotype–phenotype correlation.
Our study further broadens the clinical and variant spectrum of congenital and early-onset SPTBN4-related disorder. With the accumulation of data on βIV-spectrinopathies, it seems rational that SPTBN4 genetic testing should also be considered in patients with early-onset hypotonia, motor developmental delay, and intellectual disability, especially in the presence of axonal neuropathy, deafness, or pathological discharge patterns on the EEG.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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