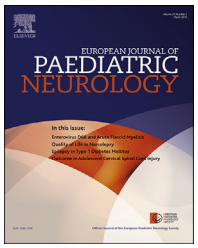
Official Journal of the European Paediatric Neurology Society



Original article

Characteristic clinical and ultrastructural findings in nesprinopathies

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ARTICLE INFO

Article history:
Received 25 August 2018
Received in revised form
18 December 2018
Accepted 22 December 2018

Keywords:
Abnormal thumb
SYNE 1
Nesprin
Nuclear envelope
Lobulated nuclei
Schwan cell nuclei
Ataxia

ABSTRACT

Aims: To define the neurological and neuropathological alterations caused by *SYNE1* mutations.

Methods: We describe 5 patients (3 males, 2 females; age 3–24 years) from 3 families. The diagnostic work-up included three muscle biopsies and two nerve biopsies in three of the cases.

Results: Three different phenotypes were discerned. Two patients showed progressive ataxia, mental retardation, neuropathy and radially deviated thumbs (spinocerebellar ataxia, SCAR, type 8 phenotype). Two patients had mild congenital myopathy with restrictive lung disease, clubfeet and thumb anomalies (myopathic arthrogryposis). One patient had congenital myopathy with dilated cardiomyopathy and adducted thumbs (Emery-Dreifuss Muscular Dystrophy, EDMD, type 4). Light microscopy of the three muscle biopsies revealed chronic non-necrotizing myopathy without rimmed vacuoles in all cases combined with neurogenic atrophy in one case. The two nerve biopsies showed predominantly axonal neuropathy with demyelinating features. Nuclear alterations, most notably lobulation and focal widening of the space between inner and outer leaflet of the nuclear envelope, were a prominent consistent feature of myonuclei and Schwann cell nuclei in each of the three muscle specimens and one nerve specimen that could be examined by electron microscopy.

Conclusion: Thumb abnormalities and nuclear envelope alterations are characteristic for *SYNE 1* mutations. Schwann cell nuclei are affected, indicating that such nuclear envelope changes in glial cells contribute to the neurodegenerative phenotype in human nesprinopathies.

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https://doi.org/10.1016/j.ejpn.2018.12.011
1090-3798/

1. Introduction

Nesprins are critical for the structural integrity of the nucleus and cytoskeleton and for proper signal transduction across the nuclear envelope (NE).[1] Many of the roles of nesprins, including functions in nuclear positioning, cell division and organization of the cytoskeleton are likely indirect effects reflecting its core function in connecting the chromatin and nucleoskeleton to the cytoskeleton.[2] Analysis of nesprin expression during muscle development revealed several isoforms. Two of the shortest alpha isoforms, nesprin-1-alpha-2 and nesprin-2-alpha-1, were found almost solely in cardiac and skeletal muscle in humans.[3] KLNes1g, a KASH-LESS isoform, is specifically expressed in the cerebellum in mice.[4]

SYNE1 mutations lead to a number of diverse diseases including Emery Dreifuss-like muscular dystrophies (EDMD), myogenic arthrogryposis and progressive spinocerebellar ataxia (SCAR).[5] However, pathogenicity with clear genotype–phenotype correlation is thought to be established in case of the proximal homozygous *SYNE 1* variants causing SCAR8[6] and of the heterozygous missense variants in *SYNE 1* close to the KASH domain which causes EDMD type 4.[7]

Diagnostic work-up in nesprinopathies is difficult because nesprinopathies have been lacking specific clinical clues up to now. There is no consistent prominent defect in nuclear envelope protein distribution detectable by immunohistochemical staining[8] and common ultrastructural defects have not been defined so far. At times when genetic analysis still depended on Sanger sequencing, the exceptionally large size of the *SYNE* genes posed a major problem, with *SYNE1* (OMIM 608441) containing 147 exons and spanning approximately 550 kb, and *SYNE2* (OMIM 608442) containing 115 exons and spanning approximately 370 kb. Nowadays, diagnosis by next generation sequencing is hampered by the frequent occurrence of sequence variants of unknown significance.

2. Methods

Five patients from three families were recruited from our pediatric neuromuscular outpatient clinic during a period of 12 months. After multi-gene panel genetic analysis had uncovered *SYNE1* mutations in four patients, the available nerve and muscle biopsy specimens were extensively re-evaluated. A salient history and focused neurological examination was performed by a neuropediatrician; prior neuroimaging studies of the brain were reviewed. One patient was diagnosed by neurological examination and specific muscle biopsy findings, then genetic analysis was performed to confirm the diagnosis.

2.1. *Standard protocol approvals, registrations, and patient consents*

The study was approved by the Ethics Committee of the University Essen (17-7518-BO). Written informed consent was obtained from all participants.

2.2. *Biopsy work-up*

Muscle biopsies had been taken from patient 1, 2 and 5 and nerve biopsies from patient 2 and 5. Serial cryosections (5 μm) of transversely-oriented muscle blocks were stained according to standard procedures with hematoxylin and eosin (H&E), Gömöri trichrome (GT), oil red O, adenosine triphosphatase (preincubation at pH 4.3 and 9.4), and nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR). Serial sections were incubated with a rabbit polyclonal anti-nesprin-1 antibody (Abcam AB5250) at 1:50 dilution. Sections were then incubated with a fluorescently labelled secondary Alexa 488 donkey anti-rabbit IgG antibody (Molecular Probes) and with 4′,6-diamidino-2-phenylindol dihydrochloride (DAPI; Carl Roth, Karlsruhe, Germany). Microscopy was performed using a Zeiss Axioplan epifluorescence microscope and a Zeiss Axio Cam ICc 1.

Glutaraldehyde-fixed specimens were processed for ultrastructural examination by standard procedures. The tissue was post-fixed in 1% osmium tetroxide and embedded in Epon 812. Semithin sections for light microscopy were stained with toluidine blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined using a Philips CM10 transmission electron microscope as described.[9]

Sural nerve biopsies were obtained from patients 2 and 5, but only in case of patient 5 a resin block was available for ultrastructural examination by standard procedures as described above.

2.3. *Genetic analysis*

Multi-gene panel analysis was performed using an Illumina MiSeq system and subsequent SIFT algorithm analysis which predicts whether an amino acid substitution affects protein function. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through PSI-BLAST.

3. Results

3.1. *Patient cohort*

Patient 1 was a 3-year-old boy, the fifth child of the eighth pregnancy from a consanguineous family with several marriages of closely related family members. Several relatives in the family of the father died in early infancy due to cardiomyopathy. The index patient showed postnatal arthrogryposis, clubfeet and muscular hypotonia. At 6 weeks of age, echocardiography revealed a dilated cardiomyopathy. Because of swallowing problems, the patient needed tube feeding. Neurological examination revealed delayed motor development, absent reflexes and adducted thumbs (Fig. 1A). Creatine kinase (CK) and motor and sensory nerve conduction studies were normal. A biopsy of the right vastus lateralis muscle was obtained during operative correction of the clubfeet at the age of 7 months.

Patient 2 was a 22-year-old woman with mental retardation and severe ataxia. She was the second of 5 children of a

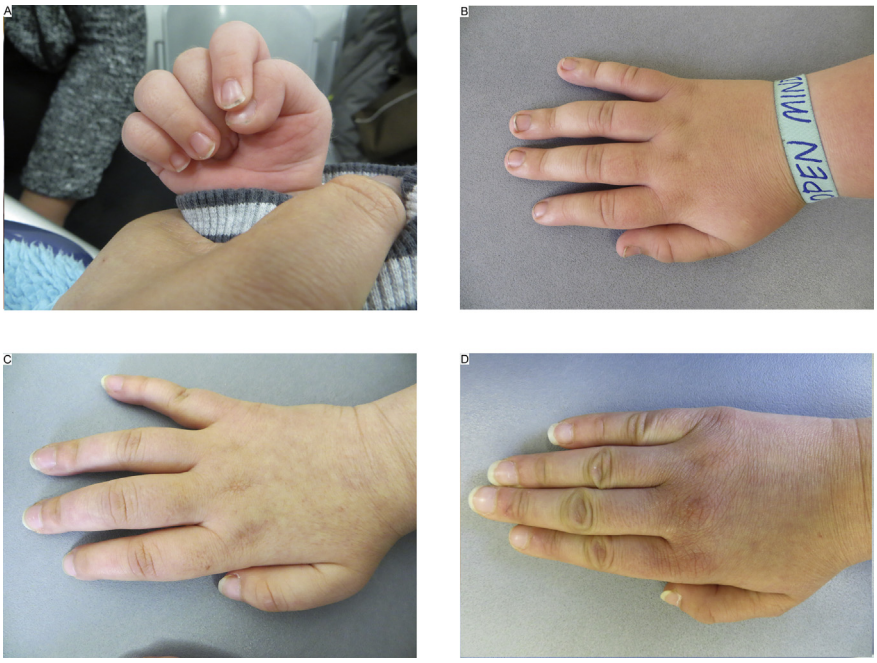

Fig. 1 – Thumb anomalies of four patients. Patient 1: Right hand at the age of eight months showed an adducted thumb (A). Patient 4: Right hand at the age of twelve years showed a radially deviated thumb (B). Patient 5 died at the age of twelve, a picture of his hand is not available. Patient 2: Right hand at the age of 19 years showed mild atrophy of the small hand muscles due to neuropathy. The thumb is radially deviated (C). Patient 3: Right hand at the age of 22 years showed a radially deviated thumb (D).

consanguineous family. First symptoms (dysarthria and unstable gait with stumble) occurred at the age of 3 years. She was non-ambulatory with severe proximal muscular weakness for two years. Cranial magnetic resonance imaging (cMRI) at the age of 12 years revealed a hypoplastic cerebellum. CK was slightly elevated (314 U/l, <180). X-ray of the right hand (performed because of short stature) showed radially deviated thumbs (Fig. 1C). At the age of nine years motor and sensory nerve conduction studies were normal. Five years later motor nerve conduction studies revealed reduced amplitudes and slightly reduced conduction velocities of 41–44 m/s (normal age-adjusted values 48–51 m/s) for the median and tibial nerves indicative of a mild axonal neuropathy. At that time neurological examination showed severe gait abnormalities with foot drop and ataxia, dysmetria and intention tremor. Hyperreflexia and myoclonus of the lower limbs indicated an upper motor neuron involvement. A combined muscle and nerve biopsy obtained at the age of 15 years revealed neuropathy and neurogenic muscular atrophy.

Patient 3 was a 24-year-old woman. She is the older sister of patient 2 and had similar symptoms with disease manifestation by an unstable gait at the age of 12. She showed a milder disease course suffering from mild mental retardation, ataxia and radially deviated thumbs (Fig. 1D). Creatine kinase (CK) was normal. At the age of 12 years motor and sensory nerve conduction studies revealed reduced amplitudes and reduced conduction velocities of 23–38 m/s (normal age-adjusted values 48–51 m/s) for the median and tibial nerves indicative of a mild demyelinating neuropathy.

Patient 4 was a 14-year-old boy who presented postnatally with generalized muscular hypotonia and clubfeet, sucking and swallowing difficulties in the first year, delayed motor development (free walking not before 3 years), and normal speech and cognitive development. By neurological examination, proximal weakness, a rigid spine, generalized muscular hypotonia, shortened and radially deviated thumbs were found (Fig. 1B). Motor and sensory nerve conduction studies were normal. CK was slightly elevated (311 U/l; <180). There was restrictive ventilatory dysfunction with weakness of the diaphragm; there is currently no need for a non-invasive or invasive ventilation.

Patient 5 was the older brother of patient 4; their parents are non-consanguineous. He died after an operative straightening of the spinal column at the age of 12 years due to multi-organ failure after bleeding problems. This patient showed a disease course similar to that of his brother. At the age of 11 years he needed non-invasive nocturnal ventilation. Muscle biopsy obtained at the age of five years revealed chronic myopathic alterations. Nerve biopsy obtained at the age of 10 showed a predominantly axonal neuropathy combined with moderate demyelination. Repeated motor and sensory nerve conduction studies were normal. CMRI at the age of five years showed normal findings.

3.2. *Muscle and nerve biopsy features*

Patient 1 (EDMD 4 phenotype) showed a mild dystrophic pattern (Fig. 2A and D) and absent nesprin immunostaining (Fig. 2G). Electron microscopy (EM) revealed lobulated myonuclei with irregularly expanded spaces between inner and outer nuclear membranes and focal condensation of chromatin predominantly at the NE (Figs. 3A and 4D).

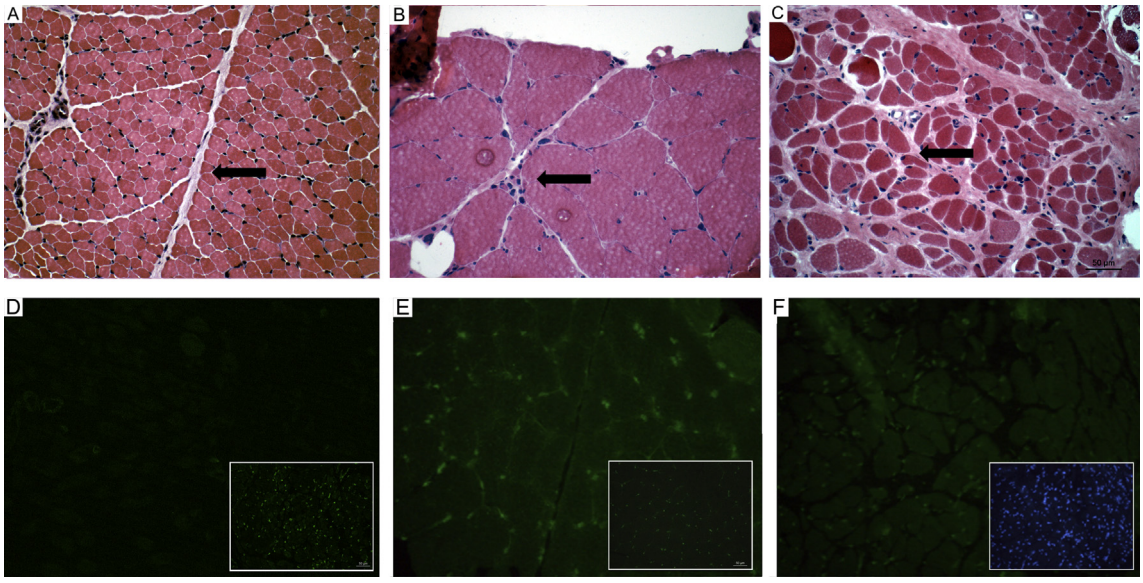

Fig. 2 – Light microscopy findings (Hematoxylin and eosin (HE), ×200) of three patients. Patient 1: Mild dystrophic pattern with increased endomysial and perimysial fibrosis, a small range of fiber size variation and basophilic and regenerating fibers (A). Absent nesprin expression (D) at the nuclear envelope compared to normal control (small window). Patient 2: Neurogenic pattern with atrophic, rounded fibers and secondary myogenic changes (central nuclei) (B). Normal nesprin expression (E) at the nuclear envelope compared to normal control (small window). Patient 5: Chronic myopathic pattern with a huge range of fiber size variation, atrophic fibers and considerable endomysial and perimysial fibrosis (C). Reduced nesprin-stained nuclei (F) in comparison to DAPI-stained nuclei (small window).

Patient 2 (SCAR8 phenotype) showed a neurogenic pattern with wide caliber spectrum and several groups of flattened or angular atrophic muscle fibers (Fig. 2B and E) and normal nesprin immunofluorescence (Fig. 2H). By EM, lobulated subsarcolemmal and non-subsarcolemmal myonuclei as well as condensation of chromatin at the NE were found (Figs. 3B and 4E). Nerve biopsy obtained at the age of 15 years showed axonal damage combined with minor unspecific lymphocytic infiltrates. Unfortunately, a resin block for ultrastructural examinations was not available.

The biopsy of Patient 5 (myopathic arthrogryposis phenotype) contained artificially altered muscle fibers; however, muscular atrophy as well as perimysial and endomysial fibrosis were clearly present (Fig. 2C and F). Immunofluorescence showed reduced nesprin expression (Fig. 2I). Ultrastructural findings included lobulated nuclei and focal condensation of chromatin (Figs. 3C and 4F).

The nerve biopsy obtained from this patient (Fig. 4) revealed considerable loss of myelinated and unmyelinated nerve fibers and several clusters of regenerating nerve fibers. Schwann cell onion bulb formations were indicative of a demyelinating component. Many Schwann cell nuclei showed irregular outfoldings of the outer leaflet of the nuclear envelope, some of which contained pleomorphic, presumably autophagic material.

3.3. *Genetic analyses*

3.3.1. *Patient 1 (EDMD type 4 phenotype)*

Sequencing of *SYNE1* (NM_182961) in the index patient revealed a variant of unclassified significance (class 3 according to ACMG): c.26278G>A:p.(Gly8760Arg), heterozygous. This putative missense variant would result in a replacement of a highly conserved glycine to arginine. It is predicted to be pathogenic according to the SIFT algorithm and is located in the KASH (Klarsicht-ANC-Syne homology) proteins domain (Fig. 5). The variant was inherited from the mother. Another variant NM_033071:c.-46C>T was found in the 5′- untranslated region which has not been described in a database before. Sequencing of *SYNE2* did not reveal any variants suspected to be pathogenic (class 3, 4, or 5 according to ACMG).

3.3.2. *Patient 2 and 3 (SCAR 8 phenotype)*

Sequencing of *SYNE 1* (NM_182961) revealed a homozygous nonsense variant in both affected sisters: c.20818_20819ins13:p.Ser6940*. Homozygosity was confirmed by segregation analysis. This variant leads to a frame-shift with subsequent stop-codon and has not been found by us in any database or in the literature. It affects the spectrin repeat – spectrin alpha-actinin protein-domain – and therefore may lead to a truncated protein without KASH domain (Fig. 5); however, it is also possible that protein expression is abolished by nonsense-mediated decay (NMD).

3.3.3. *Patient 4 and 5 (myopathic arthrogryposis phenotype)*

Sequencing of *SYNE 1* (NM_182961) revealed a homozygous stop mutation in both affected brothers: c.23995C>T:p.Arg7999*. Homozygosity was confirmed by segregation analysis. Again, this mutation probably leads to the loss of the KASH domain or protein expression might be abolished by NMD (Fig. 5).

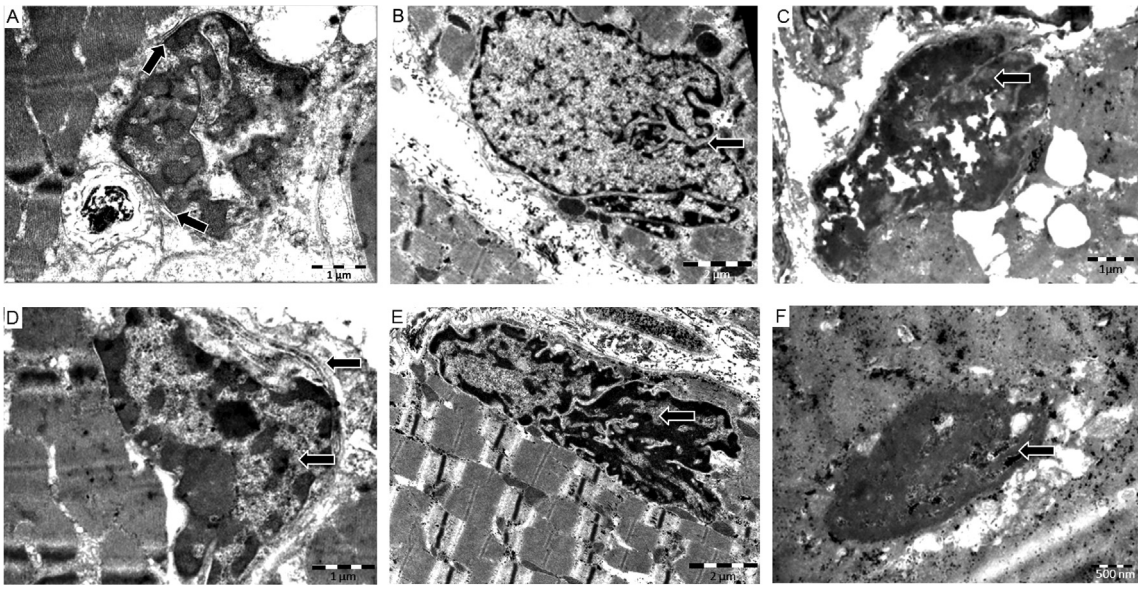

Fig. 3 – Ultrastructural findings in muscle biopsies of three patients. Patient 1: Lobulated myonucleus (A), an irregularly expanded space between inner and outer nuclear envelope membranes and granular formation of chromatin (arrows) (D). Patient 2: Lobulated myonuclei (B and E) and condensation of chromatin at the nuclear envelope (E). Patient 5: Lobulated myonuclei (C and F) in severely artificially altered tissue.

4. Discussion

In patient number 1 (EDMD 4 phenotype) the genetic analysis revealed a mutation which affects the KASH domain. The second sequence alteration was a variant with uncertain pathogenicity, but the muscle specimen showed absent nesprin immunostaining, supporting that the second variant is pathogenic. Muscle tissues only express KASH-containing isoforms of nesprin-1 which interact with the actin filaments of the cytoskeleton and with the nuclear envelope.[10] Weakening of this network is thought to lead to muscle disease in these patients, as a Δ/ΔKASH mice lacking the KASH-domain due to a homozygous deletion of the two last exons causes an EDMD-like phenotype.[11]

In patients 2 and 3 with a SCAR8 phenotype, the *SYNE1* mutation affects the spectrin repeat – spectrin alpha-actinin protein-domain –, probably causing dysfunction of a KASH-LESS variant of nesprin-1 giant (KLNes1g). KLNes1g is thought to coordinate molecular motors in vesicular transport. In mice, these isoforms are predominantly expressed in CNS tissues and most abundantly in the cerebellum, consistent with the SCAR8 phenotype in patients.[4] In contrast, the ultrastructural findings in myonuclei and Schwann cell nuclei seem to be a characteristic result in nesprinopathies. The muscular weakness in SCAR8 patients is thought to be a secondary phenomenon due to the nuclear mis-positioning.

The stop mutation found in patient 4 and 5 with myopathic arthrogrypotic (AMC) phenotype could lead to a loss of the KASH-domain. An autosomal recessive splice mutation (intron 136 to intron 137), which also leads to the loss of the KASH-domain in the resulting alternative splicing product, has already been identified as the cause of an AMC phenotype.[12] Nesprin-1 interacts with the cytoplasmic domain of MuSK, a critical component of the agrin receptor, which is concentrated in the postsynaptic membrane. Thus, nesprin-1 might be involved in the formation or maintenance of nuclear aggregates at the neuromuscular junction (NMJ).[13] The dysfunction of the NMJ could be an explanation for the AMC phenotype.

Adducted and/or radially deviated thumbs were consistently found in all of our patients. In case of patient 5 his parents reported similarities of hand and feet abnormalities in both brothers. Adducted thumbs show persistent in-fist posture, which normally occurs in the majority of newborns and resolves spontaneously in the first seven months of life. The mechanisms leading to this abnormality remain unclear.[14] If persistent, it was described as an important diagnostic clue in several genetic syndromes[15] and in congenital muscular dystrophies (CMD).[16] In a large multicenter study of patients with early-onset ataxia *SYNE 1* mutations were commonly associated with a multi-systemic phenotype as well as with hand and feet abnormalities.[17] These malformations indicate that, regardless of the time of onset of the first overt clinical symptoms, mutations in *SYNE 1* affect development.

Lobulated myonuclei with irregularly expanded spaces between inner and outer nuclear membranes and focal condensation of chromatin were a consistent ultrastructural feature in our otherwise heterogeneous nesprinopathy patient group. On the other hand, no consistent feature in nesprin expression in muscle nuclear envelope was detectable by immunohistochemical staining in our patients. Myonuclear lobulation in genetically confirmed nesprinopathy has been described in a recently reported case that also showed adducted thumbs and clubfeet.[18] In contrast to our ultrastructural findings, however, reduced heterochromatin deposition at the nuclear membrane was found in that case.

Normal mature human blood granulocytes have lobulated nuclei to facilitate malleability, which is required for the

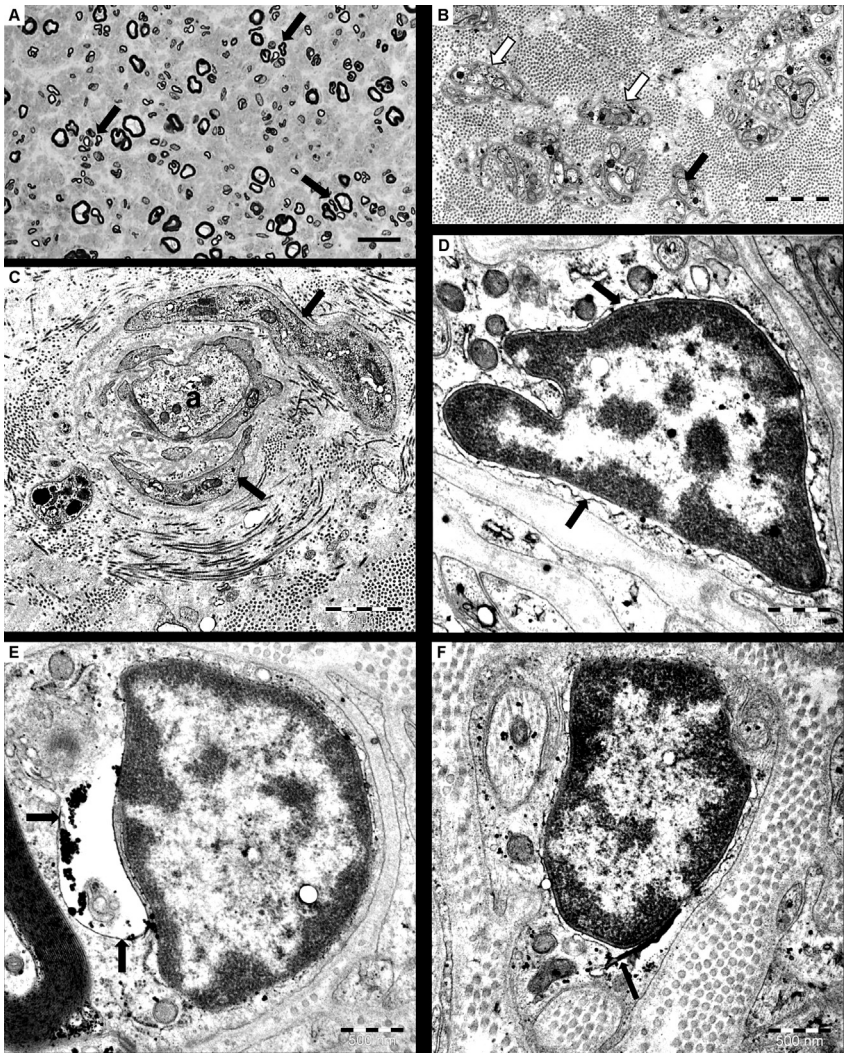

Fig. 4 – Nerve biopsy findings in Patient 5 with AMC phenotype. Patient 5: (A) Considerable loss of myelinated nerve fibers and several groups of regenerated nerve fibers (arrows). Semithin section, toluidine blue; scale bar = 30 μm. (B–F) Electronic microscopy showing marked reduction of unmyelinated axons (black arrow: remaining axon; white arrows: groups of Schwann cell processes devoid of unmyelinated axons (B). (C) Non-myelinated, probably demyelinated axon (a) surrounded by concentrically arranged surplus Schwann cell processes (Schwann cell onion bulb formation; arrows). (D) Schwann cell nucleus showing irregular evagination of the outer leaflet of the nuclear envelope. (E, F) Larger focal evaginations of the outer membranes of two Schwann cells nuclei (arrows) containing pleomorphic, presumably autophagic material.

migration in tissues.[19] The increased flexibility of the NE is probably due to the reduced expression of certain nuclear envelope components (Lamin B Receptor, lamins A/C, B1 and B2, LAP2β, and emerin) in these cells, leading to the "LINC-less" nuclear phenotype.[20] In laminopathies, changes in the nuclear structure have also been reported which, depending on the cell type or mutation, have been described as blebbing of the nuclear envelope,[21] honeycombing of the lamina, increased nuclear surface area, thickening of the nuclear lamina,[22] aberrant intranuclear foci of lamins with a decrease at the nuclear periphery, loss of peripheral heterochromatin[23] and aberrant clustering of nuclear pore complexes.[24] By analogy, the myonuclear alterations found in the present cases appear to be a logical consequence of the nesprin mutations. In unc-84 mutant muscle nuclei, irregularly expanded spacing between inner and outer nuclear envelope membranes could be found, indicating that Sad1p and UNC-84 and KASH bridges play a role in maintaining NE architecture under mechanical strain [25]. In the present study, similar morphological NE alterations could also be found in muscle and Schwann cell nuclei from patients with nesprinopathies.

In this context, the NE ultrastructural alterations found in Schwann cells of patient 5, harbouring a homozygous *SYNE1* stop, is of special interest regarding the function of nesprin-1 in the human nervous system. They suggest that nesprin mutations cause NE pathology not only in myonuclei but also in Schwann cells and potentially also in nuclei of other glial and neuronal cells. However, the phenotype of nesprinopathies depends on the loss-of-function of tissue specific nesprin isoforms, especially in nesprinopathies with overt CNS involvement such as SCAR8. Verification of these hypotheses requires further studies involving the CNS/glial cells.

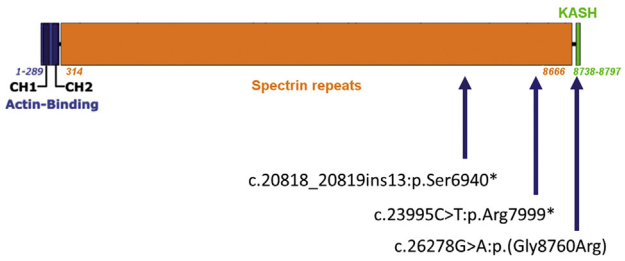

Fig. 5 – Graphical picture of the mutations related to the SYNE1 domains. Patient 1 (EDMD phenotype): c.26278G>A:p.(Gly8760Arg) with a putative missense variant is located in the KASH (Klarsicht-ANC-1/Syne homology) proteins domain (green). Patient 2 and 3 (SCAR8 phenotype): c.20818_20819ins13:p.Ser6940* affects the spectrin repeat – spectrin alpha-actinin protein-domain (orange). Patient 4 and 5 (myopathic arthrogryposis phenotype): c.23995C>T:p.Arg7999* leads to the loss of the KASH domain. The N-terminal actin-binding domain contains two calponin homology domains (CH1 and CH2) (blue); modified from Mademann, 2016.[17] (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5. Conclusion

We consistently found thumb abnormalities and ultrastructural alterations of myonuclei in patients with *SYNE 1* mutations. Careful inspection of the hands and ultrastructural examination of available muscle or nerve specimens is therefore recommended to be part of the diagnostic workup of patients with suspected nesprinopathy, in particular in those cases that are associated with *SYNE1* variants of unclear pathogenicity. In addition, we found considerable NE pathology not only in myonuclei but also in Schwann cell nuclei, indicating that such NE alterations are a general feature of nesprinopathies which also affect glial and potentially neuronal cells.

Funding

This work was supported by grants by the Interdisciplinary Centre for Clinical Research Aachen (IZKF Aachen) (N1-1, N5-3), the German Charcot-Marie-Tooth Disease Network (BMBF-CMT-Net; Funds 01GM1511D) and the German Myopathy Society (DGM) to JW.

Contributor's statement

HK conceptualized and designed the study, drafted the first version of the manuscript, interpreted results and was principally responsible for the final content.

AA carried out the initial genetic analysis and interpretation of results, contributed to the discussion, reviewed and revised the manuscript.

OS enrolled patients and critically reviewed the manuscript.

Eva N-J, IK and WP analyzed data and interpreted results.

JW carried out the initial analysis, conceptualized and designed the study, reviewed and revised the manuscript for important intellectual content.

US carried out the initial analysis, conceptualized and designed the study, reviewed and revised the manuscript for important intellectual content.

All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Conflict of interest

None declared

Acknowledgements

Dr. Ulrich Korn critically read the manuscript for linguistic corrections.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpn.2018.12.011.

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