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A recurrent ACTA1 amino acid change in mosaic form causes milder asymmetric myopathy

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ABSTRACT

We describe three patients with asymmetric congenital myopathy without definite nemaline bodies and one patient with severe nemaline myopathy. In all four patients, the phenotype had been caused by pathogenic missense variants in *ACTA1* leading to the same amino acid change, p.(Gly247Arg). The three patients with milder myopathy were mosaic for their variants. In contrast, in the severely affected patient, the missense variant was present in a *de novo*, constitutional form. The grade of mosaicism in the three mosaic patients ranged between 20 % and 40 %. We speculate that the milder clinical and histological manifestations of the same *ACTA1* variant in the patients with mosaicism reflect the lower abundance of mutant actin in their muscle tissue. Similarly, the asymmetry of body growth and muscle weakness may be a consequence of the affected cells being unevenly distributed. The partial improvement in muscle strength with age in patients with mosaicism might be due to an increased proportion over time of nuclei carrying and expressing two normal alleles.

1. Introduction

Mutations in the gene for skeletal muscle α-actin 1 (*ACTA1*), expressed in skeletal muscle tissue, are a common cause of congenital myopathies. *ACTA1* mutations often lead to severe congenital nemaline myopathy (NM) but may, in some instances, cause much milder muscle weakness [1]. Depending on the definitions used, *ACTA1*-caused myopathies may, besides NM [2], be termed: actin myopathy, congenital fibre type disproportion [3], cap myopathy, core-rod myopathy [4], zebra body myopathy [1,5], intranuclear rod myopathy [6], slowly progressive scapuloperoneal myopathy with late respiratory involvement [7], and distal NM [8]. The histopathological spectrum correspondingly comprises nemaline bodies, fibre-type disproportion, actin filament aggregates, core structures, caps, zebra bodies, and, very rarely, dystrophic features [5,7–11].

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Of more than 200 disease-causing variants in *ACTA1,* 85 % have been identified in NM patients. Subsequently, it has been estimated that 23 % of NM patients have pathogenic variant(s) in *ACTA1* [11,12]. The vast majority (approximately 90 %) of disease-causing *ACTA1* variants are dominant, of which 85 % are *de novo* variants. Ten percent of the variants identified were inherited in a recessive pattern [9].

Several families have been described to harbour a causative *ACTA1* variant presenting with confirmed somatic or inferred gonadal mosaicism. There are at least five families in which one healthy parent of the affected offspring has been found to have low-grade mosaicism for an *ACTA1* variant in their somatic cells [2,4,9,11,13,14]. Three articles describe families where more than one affected child was heterozygous for a dominant pathogenic *ACTA1* variant not identified in parental blood samples. In these cases, one of the unaffected parents in each family was inferred to be a gonadal mosaic [2,15,16]. In addition, four patients were previously described with low-grade somatic mosaicism for *ACTA1* missense variants, causing myopathies with varying clinical features [17,18].

Here, we describe three previously unpublished cases of asymmetric myopathy caused by a pathogenic *ACTA1* missense variant in mosaic form. We also summarise the findings in a patient with NM causing early death due to the same, previously published amino acid change in ACTA1 in a *de novo* constitutional form. We compare the clinical features of these patients and the courses of their disease [9].

2. Patients

2.1. Ethics approval and consents to participate

The study has been approved by the Ethics Committee of the Children's Hospital, and the Helsinki University Hospital, University of Helsinki, Helsinki, Finland (9/2021). Informed consents have been obtained from the adult patients or the parents or minor patients. Consents for the publications of the patient pictures and video have been obtained separately.

2.2. Clinical descriptions

2.2.1. Patient 1

Patient 1 (Fig. 1A-C) is a male born to healthy, non-consanguineous parents at a gestational age of 41+5 weeks, without complications and with full Apgar scores, his measurements being 3770 g, 53 cm, and 36.5 cm. Neonatally, he had feeding difficulties, which passed when breastfeeding was abandoned for bottle feeds. The boy was hypotonic and unable to hold his head up on traction. There was no family history of muscle weakness.

Because of his hypotonia, spinal muscular atrophy (SMA) was suspected. A muscle biopsy from the right quadriceps muscle at the age of 7 months was interpreted as showing considerable neurogenic group atrophy. Genetic testing for SMA and an EMG, however, gave normal results.

At 11 months of age, myopathic facies was noted, and at two years also a chest deformity. The child achieved walking at 18 months of age, having received physiotherapy from the age of three months. Speech development was normal. He had frequent and prolonged infections, which later subsided.

Echocardiography at the age of 13 years yielded normal results, whereas a repeat examination at 16 years showed a very slight mitral valve leakage. Spirometry, last done at the age of 14, showed a forced vital capacity of 80 %.

Radiological imaging at the age of 15 years revealed a scoliosis angle of 22 and a kyphosis angle of 77°. A difference in length of 2 cm had been observed in the lower limbs, and body height growth had consistently followed the +1 SD curve (one standard deviation above the mean). MRI of the muscles showed diffuse degenerative changes in all muscles, with asymmetrically smaller muscle bulk of the left lower limb (Fig. 1 D and E). Blood concentrations of creatine kinase were consistently normal, less than 100.

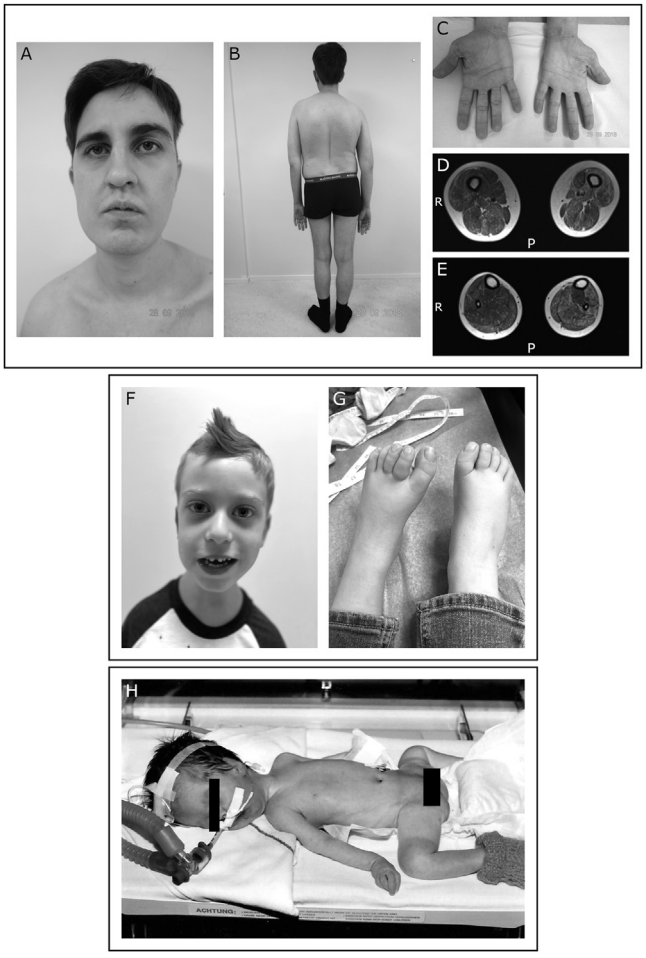

Fig. 1. The asymmetric facial expression of Patient 1 (A). His left lower limb is shorter and thinner than his right (B). The hands showed a 1.5 cm difference in breadth (C). In MRI, smaller muscle bulk was seen on the left side both at thigh (D) and lower leg (E) level. Patient 2 had elongated, myopathic facies and slight asymmetry of the facial expressions (F). The right leg was 3 cm shorter than the left one (G). Patient 4 had a very severe clinical picture at birth with extreme hypotonia, areflexia, no spontaneous movements, no independent respiratory effort, persistent bradycardia and arthrogryposis affecting all large joints (H).

A second muscle biopsy was taken from the right gastrocnemius at the age of 15 years. Red-staining clusters of cytoplasmic nemaline rods were suspected on modified Gömöri trichrome staining (Fig. 2A). Hematoxylin and eosin (H&E) and immunohistochemical MyHC slow and MyHC fast double staining showed variability of fibre size for both main fibre types, with normal fibre type distribution (Fig. 2B and C). Electron microscopy (EM) showed mini-rods and a few nemaline bodies of the usual size (Fig. 2D). The rods were positive for myotilin and alpha-actinin. A second EMG at the age of 15 was reported to show clearly myopathic findings.

On examination at the age of 16 years, he was an alert, slender young man with an asymmetric build. The right half of the face (but not the ear) was smaller, and the left lower limb was shorter and 2 cm thinner than the right one. The upper limbs were

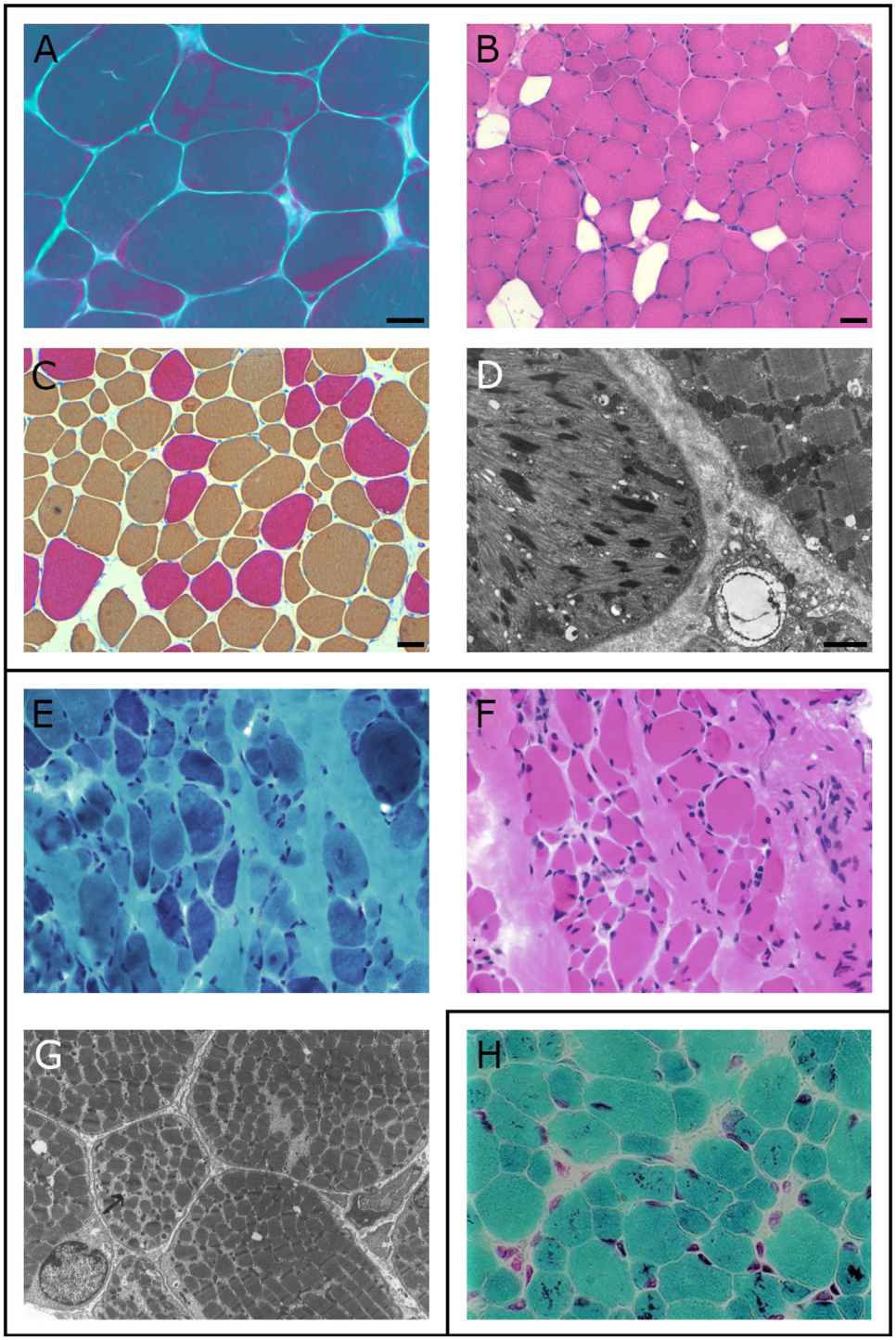

Fig. 2. In Patient 1, red-staining clusters of nemaline rods were suspected in the modified Gömöri trichrome (GTC) staining (A, scale bar 100 µm). H&E and immunohistochemical MyHC slow and MyHC fast double staining showed variability of fibre size for both main fibre types and normal fibre type distribution (B-C, scale bar 50 µm). EM showed small rods and a few nemaline bodies of the usual size (D, scale bar 1 µm). In Patient 2, GTC staining showed scattered small fibres containing possible rods (E, 400x), wide variation in fibre size with a proportion of hypotrophic fibres in the H&E staining (F, 400x) and EM image show broadened Z bands with some streaming (G). In Patient 4, GTC staining of a biopsy from 1999 confirmed the presence of nemaline rods, thus establishing the diagnosis of NM (H, 400x).

symmetric. The palate was high-arched and narrow, and the gag reflex symmetric and somewhat weak. His speech was slightly dysarthric, with difficulties especially with pronouncing "S". He had a kyphosis and a slight scoliosis of the upper part of the spine. His chest was somewhat flat and its lower part relatively narrow. The arches of the feet were low, especially the left one.

He was unable to lift his head while in the supine position. Toe-walking was almost normal, but the patient had great difficulty walking on his heels. He rose from the squatting position with a slight push of the upper limbs. His chest circumference extended a full 5 cm on inspiration, while, according to notes, the extension had previously only been 3 cm. On testing, muscle strength according to the MRC scale of 0–5 (L/R) in the facial muscles was

4/4, neck flexors 1, shoulder girdle 4/4, elbow extensors 4-/4, and flexors 4/4, wrist extensors 4-/4 and flexors 4/4, finger extensors 3.5/3.5 and flexors 4/4, hip girdle muscles 3/3, knee extensors 3.5/4.5 and flexors 4/4, ankle dorsiflexors 4/4 and flexors 3.5/4, while in the toe extensors, it was 3.5/3.5 and in the flexors 4/4. Thus, muscle strength was clearly weaker in two muscle groups on the left side than on the right. The Achilles tendon reflexes were absent, and the patellar reflexes were weak.

On re-examination at the age of 23 years in 2018, after the identification of the pathogenic variant in mosaic form, the patient was doing well and studying engineering. He did not require any assistance in the activities of daily living. By history, his muscle strength had improved by regular training, and he stumbled and fell less frequently on uneven ground than in his early childhood. The patient's cardiac, respiratory, and scoliosis status had remained largely unchanged.

The patient's facial expressions were asymmetric, with more vivid movement of the facial muscles on the smaller right-hand side (Fig. 1A). Air escaped from the left corner of his mouth on pronouncing the letter "S". The left lower limb remained shorter than the right. It was also thinner, with a difference in circumference of 1 cm in the thighs and 3 cm in the lower legs (Fig. 1B). The upper limbs did not differ in length, but the left upper arm was 5 cm, and the lower arm was 2 cm thinner than the right, with the hands showing a difference of 1.5 cm in breadth (Fig. 1C). Otherwise, the findings were largely as at the previous examination.

2.2.2. Patient 2

Patient 2 (Fig. 1F and G) was a three-year-old boy at the time of his first examination. His clinical picture and course were similar to those of Patient 1, with motor milestones delayed but reached.

The boy was born to healthy non-consanguineous parents through a Caesarean section because of breech presentation at the gestational age of 39 weeks after a normal pregnancy. The birth measurements were 3478 g, 55.5 cm, and 36.5 cm. The Apgar scores were 1/9, and the child required a few minutes of respiratory support. He had moderately severe hypotonia and spontaneous antigravity movements were present. There was head lag but no skeletal deformities or fractures. At the age of 6 months, fundoplication and insertion of a gastric tube were necessary, and orchiopexy was done at the same time. At the age of 8 months, cardiac ultrasound examination showed normal findings. The boy was referred for expert evaluation because of failure to thrive, hypotonia, speech disturbance, and delayed achievement of motor milestones.

A muscle biopsy from the vastus lateralis at the age of 5 months showed scattered small fibres containing possible rods (Fig. 2E) in Gömöri trichrome stained sections. HE staining showed a wide variation in fibre size, with a proportion of muscle fibres being hypotrophic for age (Fig. 2F). Fibre type distribution was normal, but type 1 fibres were smaller than type 2 fibres. The EM showed broadened Z bands with some streaming but no definite nemaline bodies (Fig. 2G). Some muscle fibres showed loss of thick filaments but no dystrophic features.

The boy achieved crawling at the age of 19 months and took his first steps at 29 months. Speech development was delayed also, with the first word spoken at the age of 24 months. At the age of three years, axial, proximal, and distal, muscle weakness was symmetrical, with an overall weakness grade of 3+ to 4 on the MRC scale of 0–5.

When last seen at the age of 5 years, the boy's muscle strength had improved, and he was fully ambulatory without support. He was able to climb up a flight of stairs unassisted and get up from a prone position using the Gowers manoeuvre. Fine motor skills were normal. He had some dysarthria, while cognitive skills appeared normal, even advanced. He remained G-tube-dependent but had started taking small bites and chewing.

Muscle weakness at the age of 5 years was asymmetric, with the muscles on the left side being stronger than on the right (as appendix, Video A) and with elongated, myopathic facies and slight asymmetry of the facial expressions (Fig. 1F). On the MRC scale (L/R), neck flexion and extension were 4, elbow flexion 4/3, and knee extension 4/3. The right leg was 3 cm shorter than the left one (Fig. 1G); otherwise, the length measurements of the limbs, including hands and feet, were symmetric.

2.2.3. Patient 3

Patient 3 was the third of four children of parents of Ashkenazi Jewish and Balkan Jewish ancestry. No other family members were known to have muscle weakness; the parents and the three siblings were reported healthy.

Pregnancy was uneventful, fetal scans giving normal results. The child was born at 41 weeks of gestation through an emergency Cesarean section because of decreased fetal movements. Birth weight was appropriate for gestational age (3525 g).

He was severely hypotonic, lacking spontaneous muscle movements and newborn reflexes. During the first three weeks of life, feeding was through a nasogastric tube. In infancy, he had recurrent pneumonias. Serum concentrations of creatine kinase were normal. Brain MRI neonatally gave normal findings. An echocardiogram at the age of 16 showed normal-borderline systolic function of the left ventricle, and echocardiography yielded normal results.

Muscle biopsy at eight months of age showed congenital myopathy with fibre type disproportion and no nemaline bodies according to the pathology report.

The patient never acquired independent sitting or walking. He was wheelchair-dependent and required BiPAP ventilator support during sleep. Swallowing and feeding were normal, as was intelligence.

Scoliosis appeared at the age of 3 years, requiring surgery at the age of 15 years. At the age of 16, he was admitted for genetic evaluation because of muscle weakness.

On neurological examination at the age of 20 years, he was noted to have myopathic facies, asymmetric facial weakness (right>left), high, narrow palate, very low muscle tone, and absent tendon reflexes. He was able to raise his right hand against gravity, while weakness in the legs was more pronounced on the right side. Ophthalmological examination showed high myopia in both eyes (last reported −4 in both eyes).

2.2.4. Patient 4

The pathogenic *ACTA1* variant of Patient 4 (Fig. 1H) has been published previously [9]. The female patient was born at 35 gestational weeks after a pregnancy complicated by polyhydramnios, delivered by emergency Caesarean section and had birth measurements of 48 cm, 2360 g and 35 cm, and Apgar scores of 0/1. The girl had a very severe clinical picture at birth with extreme hypotonia, areflexia, no spontaneous movements, no independent respiratory effort, persistent bradycardia and arthrogryposis affecting all large joints (Figure 1 H).

The child was resuscitated, and intubated at 25 min. She remained dependent on ventilatory support and had generalised oedema. Ultrasound evaluation of the brain showed normal findings, while cardiac evaluation showed no morphological abnormality. EMG showed no signs of upper motor neuron involvement.

Muscle biopsy at the age of 2 months yielded the diagnosis of NM (Fig. 2H). During the neonatal period, the child remained immobile, and slowly progressive contractures developed in the hips, knees, ankles, elbows, and wrists. After the first month,

the infant was moved to the pediatric intensive care unit and underwent tracheostomy. She had several bouts of ventilation-induced pneumonias and eventually died at the age of 3.5 years.

3. Methods

3.1. Massive parallel sequencing (MPS)

Blood lymphocyte-derived DNA of patients 1, 2, and 3 were analysed in custom neuromuscular gene capture panels.

The DNA analyses of Patients 1 and 2 were carried out using a NextSeq 500 (Illumina Nextera, San Diego, CA, USA) with variant annotation in Alissa (Agilent Technologies, Santa Clara, CA, USA). The analysis of Patient 1 was performed at PathWestLaboratory Medicine (Perth, Australia) and Patient 2 at GeneDx (Gaithersburg, MD, USA). The analysis of Patient 3 was performed at Invitae Corp. (San Francisco, CA, USA), as previously described [19].

Whole-genome sequencing (WGS) was performed on the blood lymphocyte DNA of Patient 2 and his parents at the HUS Diagnostic Center (Helsinki, Finland) using the Illumina NovaSeq 6000 instrument with 150-bp reads (PE300). Sequencing data were analysed using the BC|Genome software and the Germline-v2-WGS pipeline, utilising trimmomatic v0.39, BWA-MEM 0.7.15-r1140, bcQ client v1.0.45.0, the Genome Analysis Toolkit (GATK) v4.1.0.0, bcftools v1.9 and Variant Effect Predictor (VEP) v100.4 tools for variant calling and quality statistics.

3.2. Sanger sequencing

The ACTA1 gene of Patient 4 was Sanger sequenced as described in [9].

The blood lymphocyte DNA samples of the patients and their parents, and the cDNA converted from RNA extracted from the muscle biopsies of Patients' 1 and 2 were Sanger sequenced after MPS analyses.

Before MPS analysis, Sanger sequencing had been performed on DNA extracted from the saliva of Patient 1. In addition, after MPS, the cDNA extracted from a biopsy of his less affected (right) gastrocnemius muscle and buccal DNA samples from the left and right side of the mouth were Sanger sequenced (Folkhälsan Research Center, Helsinki, Finland).

3.3. Droplet digital PCR

The mosaicism was analysed and confirmed in all three patients using the QX200 Droplet Digital PCR (ddPCR) System (Bio-Rad Laboratories, Hercules, CA, USA) and FAM/HEX labelled probes targeted to the *ACTA1* variant c.739G>C (Patient 2 in HUS Diagnostic Center, Helsinki, Finland), and FAM/HEX labelled probes targeted to the *ACTA1* variant c.739G>*A* (Patients 1 and 3 at Folkhälsan Research Centre, Helsinki, Finland). DNA samples were extracted from blood. In addition, muscle-derived DNA from Patient 1 was analysed. The results were analysed using the QuantaSoft Analysis Pro software (v.1.0, Bio-Rad). A comprehensive presentation of the ddPCR experiments is available in Appendix A, with the dMIQE checklist [20] and additional materials in Appendices B-C.

3.4. Array-comparative genomic hybridization

Before running the neuromuscular gene capture panel for Patient 1, a copy number variation (CNV) analysis was performed using a custom 8 × 60k Comparative Genomic Hybridization array (NM-CGH-array) (Oxford Gene IP Limited, Oxford, UK) targeting NM-causing genes [21]. The CNV analysis of Patient 2 was performed using an exon-level CGH-array targeting neuromuscular disorder genes (ExonArrayDx by GeneDx, Gaithersburg, MD, USA).

4. Results

4.1. Patient 1

The *ACTA1* variant c.739G>*A*, p.(Gly247Arg) (NM_001100.3) was detected in mosaic form in blood lymphocyte DNA with the variant allele frequency (VAF) of 19 % (134/720 reads) using a custom neuromuscular gene capture panel (Fig. 3A). The coverage for *ACTA1* was 100 % at a minimum read depth of 20x and an average read depth of 325x.

The Sanger sequence of the same DNA sample showed a lower chromatogram peak of the variant allele. Sanger sequencing performed before MPS analyses on DNA extracted from saliva had failed to detect the variant.

The Sanger sequenced cDNA derived from a biopsy of the gastrocnemius muscle of the less affected right side (Fig. 3D, P1) and buccal DNA samples from the left and right side of the mouth showed no difference in variant abundance between any of the samples. The variant was not detected in the parental DNA samples.

The mosaicism was verified using ddPCR, and the VAF in the index patient's sample was confirmed to be 15 %, and no significant difference in VAF was seen between DNA samples extracted from blood (17 %) and muscle (14 %) (Appendices A and D).

4.2. Patient 2

A different missense variant of the same nucleotide, c.739G>C, leading to the same amino acid change, was first detected in blood lymphocyte DNA in a neuromuscular disorders panel with a 100 % coverage for *ACTA1* at a minimum read depth of 10x. The VAF of the sample was 35 % (62/175 reads), and the variant was initially interpreted as heterozygous. Because of the patient's mild and asymmetric phenotype similar to that of Patient 1, mosaicism was suspected, and using WGS analysis of the patient's blood-derived DNA sample, mosaicism was confirmed, with a VAF of 20 % (6/30 reads) (Fig. 3B). The variant was not found in the parental samples. The median coverages for the samples sequenced were 31x (index), 40x (mother), and 31x (father).

The presence of the variant was confirmed using Sanger sequencing of muscle-derived DNA of the patient (Fig. 3D, P2), while it was not detected in the parents' blood-derived DNA.

The mosaicism was confirmed using ddPCR, with which the VAF in the index patient's sample was 32 % (Appendices A and D).

4.3. Patient 3

The *ACTA1* variant c.739G>*A*, the same as the one identified in Patient 1, was found in blood lymphocyte DNA using MPS of a neuromuscular gene panel. The variant was identified in 28 % (95/339) of the reads (Fig. 3C), but because the MPS pipeline was not validated for quantitative assessment of the absolute allele ratios and the variant did not meet the internal laboratory criteria for "possibly mosaic", it was reported as heterozygous. However, because of the asymmetric weakness and body growth of the patient, the possibility of mosaicism was further considered. Subsequent quantitative ddPCR analysis showed the VAF in the index patient's sample to be 24 %, indicating mosaicism (Appendices A and D).

The presence of the variant was confirmed using Sanger sequencing of blood-derived DNA of the patient (Fig. 3D, P3). The variant was not detected in the parents' DNA samples.

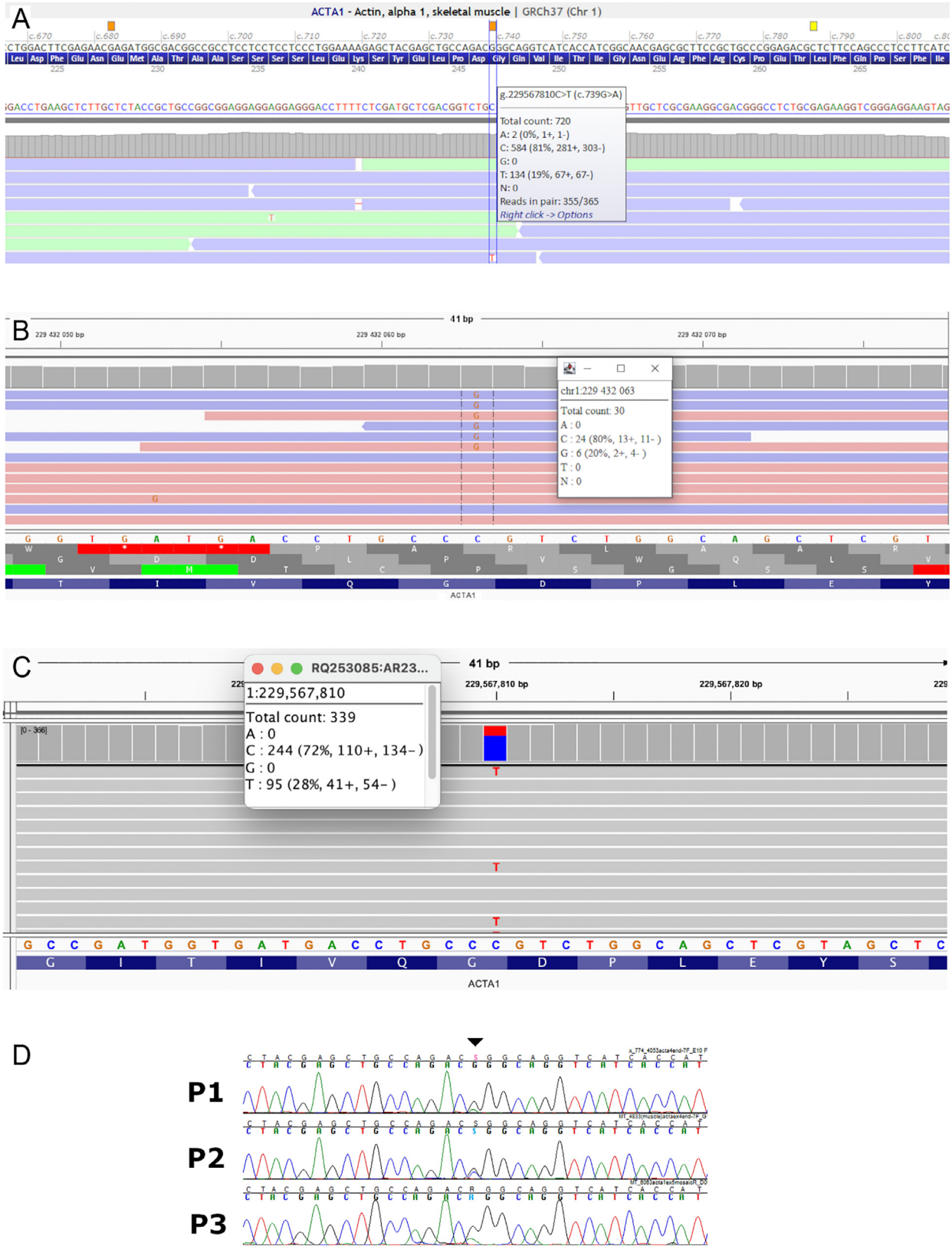

Fig. 3. The panel of Patient 1 indicated a variant allele frequency (VAF) 19 % of the c.739G>*A* variant in a DNA sample derived from blood lymphocytes (A). The IGV alignment view of the WGS data Patient 2 showing the ACTA1 c.739G>C variant in mosaic form with an indicated VAF of 20 % (B), and in Patient 3 the c.739G>*A* variant with an indicated VAF of 28 % (note that the sequence is the complement, hence the variant looks like a C>T instead of *G>A*) (C). The variants were confirmed by Sanger sequencing in Patients 1, 2, and 3 (P1-P3). (D).

Table 1
Patients with the same missense variant, p.(Gly247Arg), in ACTA1 exon 5 caused by either of the point mutations c.739G>*A* or c.739G>C.

Patient	P1	P2	P3	P4[1]
Gender	M	M	M	F
Age at latest follow-up (y)	23	5	22	Deceased at 3.5
Variant type	Mosaic	Mosaic	Mosaic	Heterozygous
Variant	c.739G>*A*	c.739G>C	c.739G>*A*	c.739G>C
VAF in MPS	Panel 18 %	Panel 35 % WGS 21 %	Panel 25–35 %	
VAF in ddPCR	In blood 17 % In muscle 14 %	In blood 32 %	In blood 24 %	?
Sanger sequencing of muscle-derived DNA	+	+	–	–
Asymmetry of muscle weakness	+	+	+	–
Asymmetry of body growth	+	+	–	–
Grade of severity*	Typical	Typical	Severe	Severe
Clinical status	Asymmetry, ambulant, studying, actively doing physical training	Asymmetry, ambulant	Asymmetry, non-ambulant, dependent on assistance	Generalised weakness, deteriorated despite active treatment, did not acquire sitting or standing
Clinical course Nemaline rods/Z-line abnormalities	Improving Minirods, a few nemaline bodies	Improving Z-band streaming, no definite nemaline bodies	Slow, modest improvement No nemaline bodies	Deceased at 3.5 yrs Nemaline bodies present
FTD	–	+	+	?

Abbreviations: *M* = male, *F* = female, VAF = variant allele frequency, FTD = Fibre type disproportion1 published in Laing at al. 2009, *according to the recent classification of NM in Laitila & Wallgren-Pettersson 2021.

4.4. Patient 4

The pathogenic dominant *de novo ACTA1* missense variant c.739G>C of the patient was identified in heterozygous form using Sanger sequencing and has been previously published [9].

The clinical data, as well as the histological and genetic findings are summarised in Table 1.

5. Discussion

A recurrent amino acid change, p.(Gly247Arg), previously reported in a severe (lethal) case of NM [9], was observed in mosaic form in three unrelated patients with notable asymmetry. Patients 1 and 2 showed a milder course, similar to that usually seen in the typical form of NM. Patient 3 had severe NM but showed improvement after infancy. He is currently aged 20 and uses a wheelchair and night-time ventilation. Patient 4, who was heterozygous for the same variant, had the severe form of NM, causing her early death.

The severe form is common in patients with pathogenic *ACTA1* variants. The more favourable course in Patients 1, 2 and 3 is probably explained by their mosaicism and a higher proportion of nuclei expressing the normal actin protein. In line with this is the fact that the same alteration in its constitutional form in Patient 4 caused severe NM. This is supported by reports of gene dosage effects in previous papers, with more favourable outcomes in patients expressing higher quantities of normal actin [1,18,22,23].

Patients 1, 2, and 3 also showed improvement in muscle strength with age. This is likely due to an increased proportion over time of nuclei carrying and expressing the normal allele. Furthermore, Patient 1 reported a clear benefit of exercise, which is in line with another *ACTA1* patient with NM described by Ilkovski and co-workers [24,25].

As muscle weakness in NM is usually symmetric, but was asymmetric in Patients 1, 2, and 3, their asymmetric weakness is likely due to their mosaicism. The asymmetric body growth and muscle weakness of the patients described in this article was paralleled by the description by Lornage and co-workers of two patients with somatic mosaicism for *ACTA1* pathogenic variants and evident asymmetry of both weakness and body growth [18]. In the current series, the medical records gave no indication of asymmetry being present at birth, while asymmetric strength and body proportions were documented later, suggesting that the asymmetry arose over time. However, as the data are derived from medical records viewed retrospectively, this is not possible to establish with certainty.

Normal growth of the skeleton is dependent, in part, on normal muscle strength in muscles exerting force on the growing bones. Congenital myopathies causing early weakness of the tongue result in the palate becoming high-arched, the mouth small, and the jaw underdeveloped and often locked. The ribs are often thin also, which is thought to be secondary to intercostal muscle weakness [26,27]. Furthermore, infants with SMA, who have strong diaphragms but weak intercostal muscles, have bell-shaped chests, while those with weakness of both the intercostal muscles and the diaphragm, such as infants with NM, often have flat chests [27,28], exemplifying that early muscle weakness does shape the growth of the skeleton [29].

The less pronounced abnormalities at muscle biopsy in the three mosaic patients, with a less evident presence of nemaline bodies, may also be due to their mosaic patterns of cell abnormality.

It is known that the grade of mosaicism may vary between different tissues, between corresponding tissues on different sides of the body, and even within the same tissue [13,30,31]. In cases in which clinical indicators suggest mosaicism or where no disease-

causing variant has been found despite extensive mutational analyses, further mutational analyses should be conducted on tissue suspected to be affected by mosaicism. Somatic variants arising in the later stages of embryonic development may be present in only a few tissues.

As the more recent sequencing methods and mutation analysis are more sensitive than the previous ones, monogenic mosaic variants are increasingly being identified and published. Yet, the most standard MPS library preparation protocols do include an enrichment step by amplification, which may introduce sequence-specific biases and affect the reliability of the quantitative data, hampering the detection of mosaicism.

Furthermore, analysis pipelines for constitutional monogenic and oligogenic disease using MPS panels have been optimised for the detection of heterozygous or homozygous variants and are usually not validated for quantitative assessment of the absolute allele ratios. The thresholds for heterozygosity are often set by each laboratory based on internal data and may range between VAF of 25–35 %. If the variant is present in a higher proportion than the set threshold, it may be considered heterozygous, and if the VAF is below the set threshold, the variant may qualify for "possible mosaic", or if below resolution level (i.e. 5 %), escape the analysis altogether. If mosaicism is suspected clinically, the data should be reanalysed using a pipeline adjusted for the detection of somatic variants or using clinically validated quantitative methods.

By optimising the analysis, mosaic variants can be extracted from the data, provided that possible enrichment steps do not skew the allele frequency and that mosaic variants are actively sought. We suggest that the read depth should be at least 80x for a higher likelihood of detecting the lower-frequency variant. However, we suggest limiting the focus and efforts spent on reaching an exact VAF for mosaic mutations, while confirmation of the mosaicism with a second method, e.g., ddPCR is necessary. Knowledge of mosaicism for a pathogenic variant is essential for the patient and for genetic counselling, as mosaic variants may imply a less severe course of the disease than a constitutional mutation. Moreover, a mosaic variant will only be passed on to any offspring if it is present in the gonads, making the risk of recurrence small, but not totally negligible.

Our study underlines the importance of carefully considering VAF thresholds and genotype-phenotype correlation in patients with asymmetry or milder phenotype, in whom no causative variant has been identified.

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Author contributions

VLL collected the data, coordinated the manuscript writing process and the molecular genetic analyses performed in Finland. VLL drafted the manuscript together with CWP, who supervised the study and coordinated the clinical descriptions as principal investigator. Medical and clinical details were provided by the medical doctors CWP, BU, MD, RS, NO and PK. Histopathology was provided by SH, MD, NO and PB. Molecular genetic analyses were performed and verified, or provided by VLL, LS, MD, DH, KaK, KiK, MV, TLW and AS. Consulting senior investigators were KP and NGL. All authors have read, critically reviewed, and approved the final version of the manuscript.

Data availability statement

WGS data cannot be shared due to ethical, legal, or commercial restrictions. ddPCR data is available upon request.

Declaration of Competing Interest

The authors declare no competing interests. Author MV is and stockholder of Invitae, and TLW is a former employee of Invitae.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2023.11.009.

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