学位論文　博士（医学）甲

Targeted massively parallel sequencing and histological assessment of skeletal muscles for the molecular diagnosis of inherited muscle disorders

遺伝性筋疾患の分子診断における骨格筋の組織学的検討を伴うターゲットシーケンスの有用性

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ORIGINAL ARTICLE

Targeted massively parallel sequencing and histological assessment of skeletal muscles for the molecular diagnosis of inherited muscle disorders

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ABSTRACT

Background Inherited skeletal muscle diseases are genetically heterogeneous diseases caused by mutations in more than 150 genes. This has made it challenging to establish a high-throughput screening method for identifying causative gene mutations in clinical practice.

Aim In the present study, we developed a useful method for screening gene mutations associated with the pathogenesis of skeletal muscle diseases.

Methods We established four target gene panels, each covering all exonic and flanking regions of genes involved in the pathogenesis of the following muscle diseases: (1) muscular dystrophy (MD), (2) congenital myopathy/congenital myasthenic syndrome, (3) metabolic myopathy and (4) myopathy with protein aggregations/rimmed vacuoles. We assigned one panel to each patient based on the results of clinical and histological analyses of biopsied muscle samples and performed high-throughput sequencing by using Ion PGM next-generation sequencer. We also performed protein analysis to confirm defective proteins in patients with major muscular dystrophies. Further, we performed muscle-derived cDNA analysis to identify splice-site mutations.

Results We identified possible causative gene mutations in 33% of patients (62/188) included in this study. Our results showed that the MD panel was the most useful, with a diagnostic rate of 46.2%.

Conclusions Thus, we developed a high-throughput sequencing technique for diagnosing inherited muscle diseases. The use of this technique along with histological and protein analyses may be useful and cost-effective for screening mutations in patients with inherited skeletal muscle diseases.

INTRODUCTION

Inherited skeletal muscle diseases, including muscular dystrophy (MD), congenital myopathy (CMP), metabolic myopathy (MM), distal myopathy and myofibrillar myopathy (MFM), are heterogeneous diseases. Until now, most muscle diseases have been categorised according to their histological presentation and clinical phenotypes. Since 1978, our laboratory, which is a part of a referral hospital, has been providing nationwide histological diagnoses for patients with muscle diseases in Japan. Until now, we have diagnosed muscle diseases in ~15 000 biopsied muscle samples. Approximately 50% of patients with muscle diseases have inherited muscle diseases. However, genetic diagnosis is not always possible because of the diversity of disease-causing genes (~150 genes) and because of the large size of some muscle genes such as *NEB* and *TTN*. Thus far, we have only performed routine gene sequencing of small genes such as *ACTA1*, *CAPN3*, *SIL1*, *GNE* and *MTM1*. We have also performed gene sequencing of some large genes such as *RYR1*; however, this was not performed for routine diagnosis but was performed as a part of a sporadic study. Recent advances in next-generation sequencing have prompted us to use this technology for gene sequencing along with routine histological analysis for disease diagnosis.

MDs are categorised based on their clinical and histological presentation. Clinically, MDs are categorised based on the presence of progressive muscle weakness with high creatine kinase levels. Histologically, MDs are categorised based on the presence of necrotic and regenerating muscle fibres, consequential endomysial fibrosis and fat tissue infiltration.[1 2] Different types of MDs, including limb-girdle muscular dystrophy (LGMD), congenital muscular dystrophy (CMD), Emery–Dreifuss muscular dystrophy (EDMD), Ullrich CMD and Bethlem myopathy, are categorised according to their clinical phenotypes.[3–7]

Histological presentation of CMP is important for its diagnosis. CMPs are characterised by hypotonia along with various abnormalities in facial development at birth because of congenital muscle weakness. CMPs are subdivided into different types such as nemaline myopathy, central core disease, myotubular myopathy and CMP with fibre-type disproportion (CFTD)[8–11] based on their histological characteristics. For example, nemaline myopathy is diagnosed based on the presence of nemaline bodies.[12 13] Congenital myasthenic syndrome (CMS) is caused by an abnormality in neuromuscular junctions. Some patients with CMS may show phenotypes similar to those of patients with CMP.[14 15]

MMs are characterised by heterogeneous clinical symptoms such as muscle weakness, exercise intolerance or rhabdomyolysis.[16–20] Commonly, MMs are caused by defects in enzymes involved in glycogen or lipid metabolism, as evidenced by glycogen or lipid accumulation in biopsied muscle samples.

Myopathy with protein aggregation/rimmed vacuoles is a heterogeneous disease. One example is MFM, which is characterised by the presence of myofibrillar disorganisation and accumulation of protein aggregates in muscle tissue with various

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clinical phenotypes.[21 22] Therefore, histological analysis of muscle samples is important for diagnosing muscle diseases.

Until now, >150 genes have been identified to be associated with the pathogenesis of inherited muscle disorders.[23] However, these genes need to be sequenced for performing accurate molecular diagnosis. High-throughput screening of causative gene mutations has been increasingly performed because of the ever-expanding availability of next-generation sequencers. Whole-exome sequencing (WES) allows the screening of various known neuromuscular disease-related gene mutations and can potentially identify new causative genes. For example, screening of LGMD genes by performing WES has been useful for detecting candidate causative mutations in 40% of sporadic patients examined.[24] Targeted sequencing of genes involved in the pathogenesis of muscle diseases may be beneficial because it is time-effective and cost-effective, can be performed in small-sized laboratories or hospitals and provides high coverage of genes of interest. However, establishment of a comprehensive diagnostic system for screening different patients with myopathies in a diagnostic setting is challenging. In the present study, we divided patients with inherited muscle diseases into four groups based on the histological characteristics of their biopsied muscle samples and screened gene mutations in these patients. Our results showed that mutation screening by using a targeted gene panel along with histological analysis was an efficient and feasible method for diagnosing inherited muscle diseases in the clinical setting.

METHODS

Patients

The study included 188 sporadic patients who were suspected of having inherited muscle diseases based on their clinical and muscle histopathological analyses but who did not undergo molecular diagnosis. All the patients were unrelated sporadic cases. Biopsied muscle and peripheral blood samples obtained from these patients were sent to our laboratory for diagnostic evaluation between 2014 and 2015. Patients with suspected mitochondrial disease were excluded. All clinical information and samples used for diagnostic purpose in this study were collected after obtaining written informed consent from the patients.

Histochemical analysis

Skeletal muscle samples were obtained from the patients by performing an open surgery. The samples were snap-frozen in liquid nitrogen; cut into 10 µm-thick sections by using standard procedures and analysed by performing routine histochemical staining procedures, including H&E staining, modified Gomori trichrome staining, NADH-tetrazolium reductase staining, succinate dehydrogenase staining, cytochrome c oxidase staining, periodic acid-Schiff (PAS) staining, phosphofructokinase staining, myosin ATPase staining, acid phosphatase and alkaline phosphatase staining, non-specific esterase staining, acetylcholinesterase staining, Congo red staining, myoadenylate deaminase staining, menadione-linked alpha-glycerophosphate dehydrogenase staining and Oil red O staining (figure 1).

Immunohistochemical analysis

Immunohistochemical analysis of proteins associated with the pathogenesis of MDs was performed for patients who were suspected of having MDs. Immunohistochemical analysis was performed using mouse monoclonal antibodies against dystrophin C-terminus (NCL-DYS2), dystrophin rod (NCL-DYS1), dystrophin N-terminus (NCL-DYS3), α-sarcoglycan (NCL-a-SALC), β-sarcoglycan (NCL-b-SALC), γ-sarcoglycan (NCL-g-SALC), δ-sarcoglycan (NCL-d-SALC), β-dystroglycan (NCL-b-DG), utrophin (NCL-DRP2), dysferlin (NCL-Hamlet), emerin (NCL-EMERIN) (all from Novocastra Lab); merosin M-chain (MAB1922; CHEMICON International); glycosylated α-dystroglycan (VIA4-1; Upstate); caveolin 3 (C38320; Transduction Lab) and collagen type VI (63175; ICN Biomedicals). Immunofluorescence staining of collagen types IV and VI was performed as described previously[25] by using rabbit anti-collagen IV (ab6586; Abcam) and mouse anti-collagen VI (VI-26; Abnova) antibodies.

Gene selection and primer design

Multiple primer sets covering exonic and exon–intron border regions (+30 to −30) of genes associated with the pathogenesis of MDs, CMP/CMS, MM and MFM/rimmed vacuolar myopathy (see online supplementary tables S1–S4) were designed using Ion AmpliSeq Designer software (Thermo Fisher Scientific). These genes were selected using the 2013 version of the gene table of monogenic neuromuscular disorders.[26] Genes associated with the pathogenesis of channelopathies were included in the MM panel because we rarely encounter patients with this disease type. Target gene numbers for the MD, CMP, MM and MFM panels were 65, 41, 45 and 36, respectively, and target gene sizes were 502, 352, 422 and 242 kb, respectively. Coverage rates of the targets (exons and flanking regions) were 96.8%, 97.2%, 97.8% and 96.7%, respectively.

Ion PGM sequencing and data analysis

Genomic DNA was isolated from peripheral blood lymphocytes by using standard techniques. Target region was enriched using Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific). Emulsion PCR was performed using Ion PGM IC 200 Kit (Thermo Fisher Scientific). Samples were loaded onto Ion 318 Chip by using Ion Chef (Thermo Fisher Scientific) and were sequenced using Ion PGM (Thermo Fisher Scientific), according to the manufacturer's protocol. Single nucleotide changes, deletions and microinsertions were reported and were annotated using National Center for Biotechnology Information (NCBI) and University of California Santa Cruz (UCSC) reference sequences and were compared using online genome databases such as National Heart, Lung, and Blood Institute (NHLBI) exome sequencing project (ESP) with ~6500 exomes, 1000 Genomes Project, dbSNP138, Human Genetic Variation Database (HGVD) for Japanese genetic variants and Exome Aggregation Consortium. We filtered variants with an allele frequency of <0.01 in these databases. Human genome reference used for these analyses was hg19. Identified candidate mutations were validated by performing Sanger sequencing with ABI Prism 3130 DNA Analyzer (Applied Biosystems). Nomenclature of the variants was confirmed using Mutalyzer, and prediction of disease-causing mutations was assessed using MutationTaster. All transcripts used in this study are presented in online supplementary tables S1–S4.

cDNA analysis

Analysis of muscle-derived cDNA was performed for patients with splice-site mutations for whom biopsied muscle samples were still available after performing histochemical and protein analyses. Total RNA was extracted from the frozen skeletal muscle samples by using TRIzol Reagent (Thermo Fisher Scientific) and RNeasy Mini Kit (QIAquick Gel Extraction Kit (QIAGEN)), and cDNA was synthesised using Oligo(dT) 15 Primer (Promega) and SuperScript IV Reverse Transcriptase

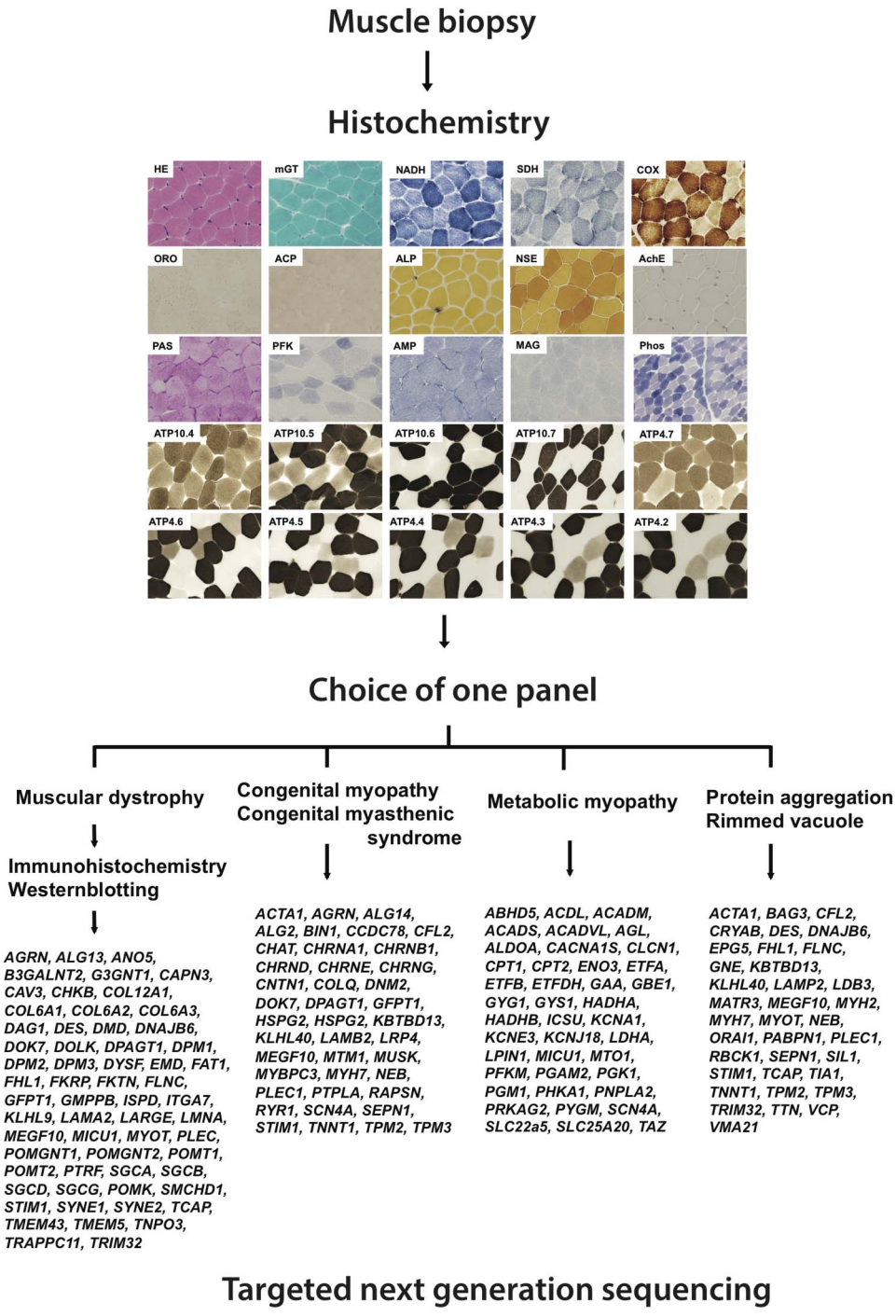

Figure 1 Muscular gene panels. Each panel includes 65, 41, 45 and 36 genes associated with the pathogenesis of muscular dystrophy (MD), congenital myopathy (CMP)/congenital myasthenic syndrome (CMS), metabolic myopathy (MM) and myopathy with protein aggregation/rimmed vacuole (MFM), respectively, and covers 96.8%, 97.2%, 97.8% and 96.7% exons and flanking regions, respectively. AchE, acetylcholinesterase staining; ACP, acid phosphatase staining; ALP, alkaline phosphatase staining; COX, cytochrome c oxidase staining; MAG, menadione-linked alpha-glycerophosphate dehydrogenase staining; mGT, modified Gomori trichrome staining; NADH, NADH-tetrazolium reductase staining; NSE, non-specific esterase staining; ORO, Oil red O staining; PAS, periodic acid-Schiff staining; PFK, phosphofructokinase staining; SDH, succinate dehydrogenase staining.

(Thermo Fisher Scientific). Primers against regions flanking splice-site mutations were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The synthesised cDNA was amplified using PCR Master Mix (Promega). PCR products obtained were extracted from agarose gel by using QIAquick Gel Extraction Kit (QIAGEN) and were sequenced directly or cloned into pCR4 vector by using TOPO-TA Cloning Kit for sequencing (Thermo Fisher Scientific) to identify the effect of these mutations. Primers used in this study are listed in online supplementary table S5.

Identification of pathogenic variants

Likely pathogenic variants were defined based on the following criteria: (1) clinical presentation and/or abnormal muscle histopathology consistent with the disease category; (2) identification of the variant at least once in patients with the same disease phenotype or categorisation of the variant as 'pathogenic' by ClinVar according to the recommendation of American College of Medical Genetics and Genomics (ACMG)[27] and/or (3) the presence of the variant as a null mutation in recessive genes, identification of the variant through a protein study (eg, identification of a defect in the encoded protein by performing immunohistochemical analysis; online supplementary figure S1) or identification of the variant as a truncating splice-site mutation based on cDNA analysis.

We used the results of prediction analysis obtained using MutationTaster, a prediction software for determining pathogenicity, as a reference. However, we did not take these results

into account because the scores may not have been accurate. Patients who had variants with unknown pathogenicity were categorised as undiagnosed.

RESULTS

MD panel

We enrolled 65 patients with suspected MDs based on the results of their clinical and muscle histopathological analyses. Immunohistochemical analyses of proteins associated with the pathogenesis of MDs were performed for all patients included in this group. The results of immunohistochemical analysis showed that these proteins were present in normal muscle samples (see online supplementary figure S1) but were absent or were stained abnormally in diseased muscle samples. The average coverage (>20 reads) in the MD panel was 98.0%.

Likely causative gene mutations were identified in *CAPN3* (1), *CAV3* (2), *COL6A1* (3,4), *COL6A2* (5,6), *COL6A3* (7,8), *DMD* (9), *DYSF* (10–13), *EMD* (14), *FKTN* (15), *LAMA2* (16–19), *LMNA* (20–22), *SGCB* (23), *SGCG* (24,25), *TRAPPC11* (26), *POMGNT2* (27, 28) and *POMT2* (29, 30) in 30 patients with MDs (figure 2 and table 1, the numbers in parentheses indicate the numbers of patients mentioned in table 1 and online supplementary figures S2–S19). Clinical findings of all the patients were consistent with the detected genotype. Relevant protein defects or dislocations were confirmed based on the results of immunohistochemical analysis (see online supplementary figure S1), which supported the genetic diagnosis in all patients, except in patients with mutations in *CAPN3*, *LMNA* and *TRAPPC11*. In addition, we performed cDNA analysis for patients 5–7 who harboured splice-site mutations in genes encoding collagen type VI and confirmed the presence of aberrant splicing that disrupted repetitive glycine residues in a triple-helical region, which is pathogenic in either gene (see online supplementary figures S6–S8).

Two patients had unreported *LMNA* variants whose phenotypes were consistent with those associated with laminopathy (see online supplementary table S5). However, these mutations did not meet our criteria. One patient with laminin alpha-2 deficiency and one patient with sarcoglycan gamma deficiency, as determined by performing immunohistochemical analysis, did not have any pathogenic variants in *LAMA2* or *SGCG* (data not shown).

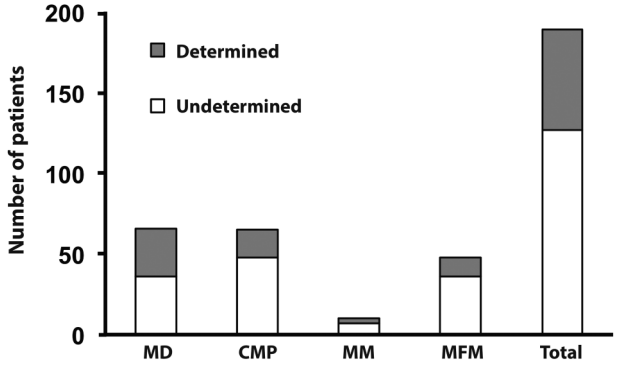

Figure 2 The total diagnostic yield was 33.0%. The diagnostic yield of the muscular dystrophy (MD) panel was 46.2%, congenital myopathy (CMP)/congenital myasthenic syndrome (CMS) panel was 26.2%, metabolic myopathy (MM) panel was 30.0% and myopathy with protein aggregation/rimmed vacuole (MFM) panel was 25.0%. Determined: likely pathogenic variants; undetermined: variants that did not meet our criteria for likely pathogenic variants.

CMP panel

We enrolled 65 patients with CMP. One patient was suspected as having CMS according to the results of electrophysiological analysis. Muscle biopsy was not performed for this patient because muscles of patients with CMS usually show non-specific changes and are not very useful for diagnosis. The average coverage (>20 reads) in the CMP panel was 98.0%.

We prioritised variants in genes that were consistent with clinical and histological phenotypes of reported patients. We identified causative gene mutations in *ACTA1* (31), *CHRNE* (32), *KLHL40* (33, 34), *NEB* (35), *MTM1* (36–38), *RYR1* (39–45), *TPM2* (46) and *TPM3* (47) in 17 patients (figure 2, table 2 and online supplementary figures S20–S26). Patient 36 had an intronic mutation in *MTM1* (c.1261-10A>G). Analysis of cDNA obtained from this patient confirmed the presence of an aberrant splicing that was reported previously[28] (see online supplementary figure S23).

We identified variants in *DNM2*, *RYR1* and *NEB* in 13 patients, which was consistent with their respective phenotypes. However, we could not describe these variants as pathogenic because they have not been reported previously or have been reported for a different phenotype (such as malignant hyperthermia). These variants are listed in online supplementary table S5.

MM panel

We enrolled 10 patients with suspected MM. One patient was diagnosed with glycogen storage disease based on glycogen accumulation, as determined by performing PAS staining (48). Two patients (patients 49 and 50) were diagnosed with glycogen phosphorylase deficiency based on the results of routine histochemical staining (see online supplementary figures S26–S28), and three patients were diagnosed with lipid storage myopathy. The remaining four patients showed non-specific clinical and histological phenotypes but were suspected of having MM, with two patients having myalgia, one patient having rhabdomyolysis and one patient having cyclic vomiting. cDNA analysis was performed for patient 48 to confirm the presence of aberrant splicing, which was similar to that reported in a patient with the same mutation[29] (see online supplementary figure S27). The average coverage (>20 reads) in the MM panel was 98.9%.

We detected causative mutations (table 3) in *AGL* (48) and *PYGM* (49,50) in three patients with glycogenosis (figure 2 and table 3) and did not detect any variants in other patients, suggesting the heterogeneous nature of the clinical phenotype.

Myopathy with protein aggregation/rimmed vacuole panel (MFM panel)

We enrolled 48 patients who showed protein aggregation, rimmed vacuoles and/or myofibrillar disorganisation in biopsied muscle samples (table 4) and identified probable causative gene mutations in *DNAJB6* (51), *GNE* (52–55), *MYH2* (56), *MYOT* (57), *SEPN1* (58), *TTN* (59, 60) and *VCP* (61, 62) in 12 patients (figure 2, table 4 and online supplementary figures S29–S34). The average coverage (>20 reads) in the MFM panel was 98.9%.

Patient 58 had one fibre with a rimmed vacuole; however, nicotinamide adenine dinucleotide (NADH) staining detected multi-minicores in this patient (see online supplementary figure S32). The clinical phenotype and muscle histopathological presentation, except for the rimmed vacuole, in this patient were consistent with a multi-minicore disease associated with *SEPN1* mutations. Patients 59 and 60 had common Japanese variants in *TTN*, which are associated with hereditary myopathy with early

Table 1 Pathogenic variants identified using the MD panel

#	Age	Sex	Ethnicity	Phenotype	IHC result	Gene	cDNA	Status	Protein	1000g	ESP6500	HGVD	ExAC	Clin var	MutationTaster	Online Supplementary figures	Reported
1	77 years	F	J	LGMD	Normal	*CAPN3*	c.1381C>T	hom	p.(Arg461Cys)	–	–	0.0022	–	–	Disease causing	2	Yes
2	19 years	F	J	CMD	Caveolin def.	*CAV3*	c.436del	het	p.(Val146Cysfs*107)	–	–	–	–	–	Polymorphism	3	No
3	23 years	F	J	UCMD	SSCD	*COL6A1*	c.841G>A	het	p.(Gly281Arg)	–	–	–	–	Pathogenic	Disease causing	4	Yes
4	1 month	M	J	UCMD	SSCD	*COL6A1*	c.1138G>A	het	p.(Gly380Arg)	–	–	–	–	–	Disease causing	4,5	No
5	5 years 6 months	M	J	UCMD	SSCD	*COL6A2*	c.801+2T>C	het		–	–	–	–	–*	Disease causing	4-6	Yes
6	3 years 5 months	F	J	UCMD	SSCD	*COL6A2*	c.955-2A>G	het		–	–	–	–	–	Disease causing	4,7	Yes
7	3 years 4 months	M	J	UCMD	SSCD	*COL6A3*	c.6283-1G>T	het		–	–	–	–	–†	Disease causing	4,8	No
						COL6A3	c.6310-2A>T	het		–	–	–	–	–	Disease causing		No
8	12 years	F	J	Bethlem	SSCD	*COL6A3*	c.5525G>A	het	p.(Gly1842Glu)	–	–	–	–	–	Disease causing	4	Yes
9	8 years 6 months	M	EG	DMD	Dystrophin def.	*DMD*	c.5530C>T	hemi	p.(Arg1844*)	–	–	–	–	Pathogenic	Disease causing	9	Yes
10	16 years	M	J	Miyoshi MP	Dysferlin def.	*DYSF*	c.755C>T	het	p.(Thr252Met)	–	–	–	–	Uncertain significance	Disease causing	10	Yes
						DYSF	c.5873C>T	het	p.(Ser1958Phe)	–	–	0.0012	–	–	Disease causing		No
11	54 years	F	J	LGMD	Dysferlin def.	*DYSF*	c.2997G>T	hom	p.(Trp999Cys)	–	–	–	0.000016	Pathogenic	Disease causing	10	Yes
12	22 years	M	J	LGMD	Dysferlin def.	*DYSF*	c.4756C>T	het	p.(Arg1586*)	–	–	–	0.000016	Pathogenic	Disease causing	10	Yes
							c.5608A>T	het	p.(Arg1870Trp)	–	–	–	–	–	Disease causing		No
13	40 years	M	J	LGMD	Dysferlin def.	*DYSF*	c.2997G>T	het	p.(Trp999Cys)	–	–	–	0.000016	Pathogenic	Disease causing	10	Yes
							c.3105C>G	het	p.(Tyr1035*)	–	–	–	–	–	Disease causing		No
14	34 years	M	J	EDMD	Emerin def.	*EMD*	c.1A>G	hemi	p.?	–	–	–	–	Pathogenic	Disease causing	11	Yes
15	7 years 10 months	M	J	FCMD	Disglycosylated aDG	*FKTN*	c.497T>C	het	p.(Leu166Pro)	–	–	–	–	–	Disease causing	12	No
16	1 years 4 months	M	J	CMD	Merosin def.	*LAMA2*	c.3747T>G	het	p.(Tyr1249*)	–	–	–	0.000008	–	Disease causing	13	No
						LAMA2	c.9085_9086del	het	p.(Thr3029Cysfs*9)	–	–	–	–	–	Disease causing		No
17	1 years 1 months	F	J	CMD	Merosin partial def.	*LAMA2*	c.2049_2050del	het	p.(Arg683Serfs*21)	–	–	–	0.000091	Pathogenic	Disease causing	13	Yes
						LAMA2	c.6513_6515del	het	p.(Val2172del)	–	–	–	–	–	Disease causing		Yes
18	42 years	M	J	CMD	Merosin def. (skin)	*LAMA2*	c.1027+1G>T	het		–	–	–	–	–	Disease causing		No‡
						LAMA2	c.3425G>C	het	p.(Gly1142Ala)	–	–	0.0023	0.000075	–	Disease causing		No
19	1 years 4 months	F	J	CMD	Merosin partial def.	*LAMA2*	c.4936G>T	het	p.(Glu1646*)	–	–	–	–	–	Disease causing	13	No
						LAMA2	c.8934_8943del	het	p.(Gly2979Valfs*11)	–	–	–	–	–	Disease causing		No
20	3 years	M	J	CMD	Normal	*LMNA*	c.94_96del		p.(Lys32del)	–	–	–	–	Not provided	Disease causing	14	Yes
21	2 years 11 months	M	J	LGMD	Normal	*LMNA*	c.810+1G>A	het		–	–	–	–	Not provided	Disease causing	14	Yes

Continued

Table 1 Continued

#	Age	Sex	Ethnicity	Phenotype	IHC result	Gene	cDNA	Status	Protein	1000g	ESP6500	HGVD	ExAC	Clin var	MutationTaster	Online Supplementary figures	Reported
22	49 years	M	J	LGMD	Normal	*LMNA*	c.1255C>T	het	p.(Arg419Cys)	–	–	–	0.000008	–	Disease causing	14	Yes
23	6 years 8 months	M	J	LGMD	All SGs def.	*SGCB*	c.753+5G>A	het		–	–	–	–	–	Disease causing	15,16	No
							c.325C>T	het	p.(Arg109*)	–	–	–	–	–	Disease causing		No
24	36 years	M	EG	LGMD	All SGs def.	*SGCG*	c.2T>C	het	p.?	–	–	–	–	–	Disease causing	17	No
						SGCG	c.787G>A	het	p.(Glu263Lys)	–	–	–	0.000025	Pathogenic	Disease causing		No
25	8 years 1 months	F	J	LGMD	All SGs def.	*SGCG*	c.320C>T	hom	p.(Ser107Leu)	–	–	–	–	–	Disease causing	17	No
26	4 years 2 months	F	TW	CMD	Normal	*TRAPPC11*	c.661-1G>T	het		–	–	–	–	–	Disease causing		Yes§
						TRAPPC11	c.2938G>A	het	p.(Gly980Arg)	–	–	–	0.000041	Pathogenic	Disease causing		Yes§
27	3 years	F	J	CMD	Disglycosylated aDG	*POMGNT2*	c.577_579del	hom	p.(Phe193del)	–	–	–	–	–	Disease causing	18	No
28	3 years 4 months	M	J	LGMD	Disglycosylated aDG	*POMGNT2*	c.758C>T	het	p.(Pro253Leu)	–	0.000077	0.0027	0.000016	-	Disease causing	18	No
						POMGNT2	c.577_579del	het	p.(Phe193del)	–	–	–	–	–	Disease causing		No
29	15 years	F	J	LGMD	Disglycosylated aDG	*POMT2*	c.869C>T	het	p.(Pro290Leu)	–	–	–	–	–	Disease causing	19	No
							c.1568A>C	het	p.(Asn523Thr)	–	–	–	–	–	Disease causing		No
30	33 years	M	J	LGMD	Disglycosylated aDG	*POMT2*	c.1568A>C	hom	p.(Asn523Thr)	–	–	–	–	–	Disease causing	19	No

p.? means protein is unknown according to hgvs nomenclature recommendation.
*c.801+1 is pathogenic.
†c.6283-2 is likely pathogenic.
‡c.1027+3A>G has been reported.
§This case.
aDG, alpha dystroglycan; Bethlem, Bethlem myopathy; CMD, congenital muscular dystrophy; def., deficiency, as determined by performing immunohistochemical staining; DMD, Duchenne muscular dystrophy; EDMD, Emery–Dreifuss muscular dystrophy; EG, Egyptian; ExAC, Exome Aggregation Consortium; FCMD, Fukuyama congenital muscular dystrophy; IHC, immunohistochemistry; J, Japanese; LGMD, limb-girdle muscular dystrophy; MD, muscular dystrophy; SGs, sarcoglycans; SSCD, sarcolemma-specific collagen deficiency; TW, Taiwanese; UCMD, Ullrich congenital muscular dystrophy.

Table 2 Pathogenic variants identified using the CMP/CMS panel

#	Age	Sex	Ethnicity	Phenotype	Gene	cDNA	Status	Protein	1000g	ESP6500	HGVD	ExAC	Clin var	MutationTaster	Online supplementary figures	Reported
31	3 months	F	J	NM	*ACTA1*	c.282C>A	het	p.(Asn94Lys)	–	–	–	–	–	Disease causing	20	Yes
32	18 years	M	EG	CMS	*CHRNE*	c.1181_1187dup	hom	p.(Glu396Aspfs*3)	–	–	–	–	–	Disease causing		No
33	2 months	M	J	NM	*KLHL40*	c.1405G>T	het	p.(Gly469Cys)	–	–	–	0.000033	Pathogenic	Disease causing	21	Yes
					KLHL40	c.1582G>A	het	p.(Glu528Lys)	–	–	–	0.000067	Pathogenic	Disease causing		Yes
34	5 months	M	J	NM	*KLHL40*	c.1405G>T		p.(Gly469Cys)	–	–	–	0.000033	Pathogenic	Disease causing	21	Yes
					KLHL40	c.1582G>A		p.(Glu528Lys)	–	–	–	0.000067	Pathogenic	Disease causing		Yes
35	9 months	F	J	NM	*NEB*	c.24681C>G	hom	p.(Tyr8227*)	–	–	–	–	–	Disease causing	21	No
36	1 year	M	?	MTM	*MTM1*	c.1261-10A>G	hemi		–			–	Pathogenic	Polymorphism	22,23	Yes
37	11 months	M	J	MTM	*MTM1*	c.1497G>A	hemi	p.(Trp499*)	–			–	Pathogenic	Disease causing	22	No
38	1 years 6 months	M	J	MTM	*MTM1*	c.1536dup	hemi	p.(Phe513Leufs*4)	–			–	–	Disease causing	22	No
39	8 years	F	J	CCD	*RYR1*	c.131G>A	het	p.(Arg44His)	–	0.000078	–	0.000010	Uncertain significance	Disease causing	24	Yes
					RYR1	c.7635G>C	het	p.(Glu2545Asp)	–	–	–	0.000009	Pathogenic	Disease causing		Yes
40	67 years	F	J	CCD	*RYR1*	c.14378T>C	het	p.(Leu4793Pro)	–	–	–	–	Pathogenic	Disease causing	24	Yes
41	4 years	M	J	CCD	*RYR1*	c.14581C>T	het	p.(Arg4861Cys)	–	–	–	–	Pathogenic	Disease causing	24	Yes
42	4 years 10 months	M	J	CCD	*RYR1*	c.14740A>G	het	p.(Arg4914Gly)	–	–	–	–	Pathogenic	Disease causing	24	Yes
43	30 years	F	J	CCD	*RYR1*	c.14741G>T	het	p.(Arg4914Met)	–	–	–	–	–*	Disease causing	24	Yes
44	3 years 1 months	F	J	CCD	*RYR1*	c.14590T>G	het	p.(Tyr4864Asp)	–	–	–	–	–†	Disease causing	24	Yes
45	7 months	F	J	CFTD	*RYR1*	c.1001G>T	het	p.(Gly334Val)	–	–	–	–	Uncertain significance	Disease causing	25	No
					RYR1	c.1186_1187inv	het	p.(Glu396Ser)	–	–	–	–	Uncertain significance	Disease causing		No
					RYR1	c.4071_4072del	het	p.(Gly1359Hisfs*16)	–	–	–	–	Pathogenic	Disease causing		No
					RYR1	c.4717C>A	het	p.(Pro1573Thr)	–	–	–	–	–	Disease causing		Yes
46	70 years	F	J	NM	*TPM2*	c.428T>C	het	p.(Leu143Pro)	–	–	–	–	–	Disease causing	21	Yes
47	9 years 5 months	F	J	CFTD	*TPM3*	c.502C>G	het	p.(Arg168Gly)	–	–	–	–	Pathogenic	Disease causing	25	Yes

*Arg >Thr and Gly are reported to be pathogenic.
†Tyr >Cys is reported to be pathogenic.
CCD, central core disease; CFTD, congenital myopathy with fibre-type disproportion; CMP, congenital myopathy; CMS, congenital myasthenic syndrome; EG, Egyptian; ExAC, Exome Aggregation Consortium; J, Japanese; MTM, myotubular myopathy; NM, nemaline myopathy.

Table 3 Pathogenic variants identified using the MM panel

#	Age	Sex	Ethnicity	Phenotype	Protein study	Gene	cDNA	Status	Protein	1000g	ESP6500	HGVD	ExAC	Clin var	MutationTaster	Online supplementary figures	Reported
48	47	F	J	Glycogenosis type III		*AGL*	c.1735+1G>T	hom		0.0005	–	–	0.000016	Pathogenic	Disease causing	26, 27	Yes
49	57	M	J	McArdle disease	Phosphorylase↓	*PYGM*	c.1531delG	hom	p.(Asp511Thrfs*28)	–	–	–	–	–	Disease causing	28	Yes
50	13	M	J	McArdle disease	Phosphorylase↓	*PYGM*	c.2128_2130del	hom	p.(Phe710del)	–	–	–	–	Pathogenic	Disease causing	28	Yes

ExAC, Exome Aggregation Consortium; J, Japanese; MM, metabolic myopathy.

respiratory failure (HMERF). These patients have not yet developed respiratory failure; however, their other symptoms and the results of muscle histopathological analysis are consistent with HMERF.

Summary of the four panels

The overall diagnostic yield was 33.0% in 188 patients. The rates for detecting the most likely causative gene mutations by the MD, CMP, MM and MFM panels were 46.2%, 26.2%, 30.0% and 25.0%, respectively (figure 2).

DISCUSSION

In the present study, we developed four targeted gene sequencing panels by using the Ion Torrent sequencing system and assessed their unbiased diagnostic yield in combination with histological and protein analyses. We separated genes into the four panels rather than combining them into a single large panel to mainly achieve cost efficiency and time efficiency. This approach also reduced labour required for interpreting data but might have overlooked known genes associated with unexpected phenotypes. However, it is essential to improve these panels because some novel muscle disease-related genes were identified after the development of these panels. The rate of genetic diagnosis varied for each panel, with the MD panel having the highest diagnostic rate (46.2%), which was comparable with or higher than that reported in a previous study involving WES.[24] MD is a heterogeneous inherited muscle disease. The most prevalent forms of MD in both children and adults in Japan are dystrophinopathy, which is caused by *DMD* mutations; myotonic dystrophy, which is caused by *CTG* expansion in the 3′ untranslated region (UTR) in *DMPK*; facioscapulohumeral MD, which is caused by the contraction of the D4Z4 repeat (a 3.3 kb macrosatellite repeat in 4q35) and Fukuyama CMD, which is caused by a retrotransposonal 3 kb insertion in the 3′UTR of *FKTN*. These diseases are usually clinically distinguishable and can be diagnosed in local hospitals by performing multiplex ligation-dependent probe amplification, PCR or Southern blotting before their evaluation at our laboratory. In the present study, most of the prevalent MDs were excluded at the routine clinical testing level. Patients with other MDs are usually categorised clinically based on the presence of LGMD, CMD or EDMD. Known gene mutations in patients with these diseases are mostly caused by single nucleotide variants or small insertions and deletions. Therefore, next-generation sequencing is a powerful tool to detect these mutations. Among 62 genes examined for MD, 66% causes the disease in a recessive manner, suggesting the presence of a loss-of-function mechanism. Therefore, immunohistochemical analysis to detect the loss of a protein is an effective method to diagnose these recessive diseases.[30 31] In the present study, 25 patients yielded abnormal results for immunohistochemical analysis, and their diagnosis was confirmed by performing molecular analysis. These results suggest that immunohistochemical analysis is very helpful for improving the precision of genetic diagnosis and should be used routinely together with genetic testing. In one patient with laminin alpha-2 deficiency and one patient with sarcoglycan gamma deficiency, which were detected by performing immunohistochemical analysis, we could not detect any pathogenic variants in *LAMA2* or *SGCG*. However, the coverage of the gene was 96.9% and 91.6%, respectively, in MD panel. This might be because of the limitation of protein analysis or technical issues associated with this method (ie, variants outside of the target region, such as promoter region; variants in homopolymers or the presence of copy number variations). Therefore,

Table 4 Pathogenic variants identified using the MFM panel

#	Age	Sex	Ethnicity	Phenotype	Gene	cDNA	Status	Protein	1000g	ESP6500	HGVD	ExAC	Clin var	MutationTaster	Online supplementary figures	Reported
51	57 years	M	J	AVM	*DNAJB6*	c.279C>G	het	p.(Phe93Lys)	–	–	–	–	–	Disease causing	29	Yes
52	42 years	F	J	DMRV	*GNE*	c.1807G>C	het	p.(Val603Leu)	0.0005	–	0.0043	0.00002	–	Disease causing	30	Yes
						c.620A>T	het	p.(Asp207Val)	0.0009	–	0.0018	0.00004	–	Disease causing		Yes
53	29 years	M	J	DMRV	*GNE*	c.188_197dup	het	p.(Glu66Aspfs2)	–	–	–	–	–	Disease causing	30	Yes
						c.1807G>C	het	p.(Val603Leu)	0.0005	–	0.0043	0.00002	–	Disease causing		Yes
54	41 years	F	J	DMRV	*GNE*	c.620A>T	het	p.(Asp207Val)	0.0009	–	0.0018	0.00004	–	Disease causing	30	Yes
						c.1807G>C	het	p.(Val603Leu)	0.0005	–	0.0043	0.00002	–	Disease causing		Yes
55	30 years	F	J	DMRV	*GNE*	c.131G>C	hom	p.(Cys44Ser)	–	–	–	–	–	Disease causing	30	Yes
56	38 years	M	J	Distal myopathy	*MYH2*	c.2414T>C	het	p.(Val805Ala)	0.0018	–	0.0093	0.00081	-	Disease causing	31	Yes
57	61 years	M	J	MFM	*MYOT*	c.179C>G	het	p.(Ser60Cys)	–	–	–	–	Pathogenic	Disease causing	13	Yes
58	41 years	M	J	AVM_scoliosis_resp failure	*SEPN1*	c.565C>T	het	p.(Arg189)	–	–	–	0.00001	–	Disease causing	32	No
						c.1574T>G	het	p.(Met525Arg)	–	–	0.0037	0.00006	–	Disease causing		Yes
59	54 years	M	J	MFM	*TTN*	c.95135G>A	het	p.(Cys31712Tyr)	–	–	–	–	–	Disease causing	33	Yes
60	46 years	F	J	HMERF	*TTN*	c.95136T>G	het	p.(Cys31712Tyr)	–	–	–	–	–	Disease causing	33	Yes
61	44 years	M	J	Myopathy	*VCP*	c.463C>T	het	p.(Arg155Cys)	–	–	–	–	Pathogenic	Disease causing	34	Yes
62	45 years	M	J	Distal myopathy	*VCP*	c.476G>A	het	p.(Arg159His)	–	–	–	–	Pathogenic	Disease causing	34	Yes

AVM, autophagic vacuolar myopathy; DMRV, distal myopathy with rimmed vacuole; ExAC, Exome Aggregation Consortium; HMERF, hereditary myopathy with early respiratory failure; J, Japanese; MFM, myofibrillar myopathy.

further analysis such as Sanger sequencing of all target exonic regions or WES may be necessary to detect these mutations. Immunohistochemical analysis is not useful in patients with some dominant gene mutations, such as patients with mutations in *LMNA*. Two unreported *LMNA* variants were identified in patients with EDMD included in our study. We could not determine their pathogenicity despite the consistency with clinical and pathological findings. Therefore, we included these patients in the undiagnosed category. It is necessary to include more patients in disease variant databases to check for shared mutations. Family analysis will be helpful to determine the pathogenicity of these variants. However, only sporadic cases were analysed in the present study.

Several patients with nemaline myopathy had variants in *NEB*; however, we could not determine whether these variants were pathogenic because most variants were unreported and the coverage of *NEB* was low due to a repeat region with high homology, suggesting the presence of other mutations in this region.[32] Adoption of a next generation sequencing approach for *NEB* is challenging for identifying variants in this region. Transcript analysis might be useful for identifying these variants. We also identified several rare variants in *RYR1* in patients with CFTD or CMP with type 1 fibre predominance. Mutations in *RYR1* are associated with these phenotypes;[33 34] however, it is unclear whether these mutations are pathogenic because there is no suitable in vitro or in vivo analytical method to prove their pathogenicity. Interestingly, patients 6 and 7 shared the same phenotype and the same rare variant in *NEB*; moreover, patients 12 and 13 shared the same rare variant in *RYR1*. Thus, collection of variant data from diseased and healthy subjects is important for determining the pathogenicity of these variants.

MM is caused by defects in enzymes involved in glycogen and lipid metabolism. In Japan, glycogen storage disease is mainly diagnosed by performing biochemical assessment of these enzymes. Therefore, such samples are rarely sent to our laboratory for genetic diagnosis. Future studies involving more patients are necessary to evaluate the diagnostic yield of the MM panel and to obtain a mutation spectrum of this disease.

Some patients with myopathies show fibres with marked accumulation of protein aggregates or rimmed vacuoles, which is a marker of autophagy. These highly heterogeneous diseases include MFM, VCP myopathy, GNE myopathy, TTN myopathy, oculopharyngeal distal myopathy, oculopharyngeal MD and autophagic vacuolar myopathy.[22 35–40] MFM has been described only recently, and not many patients with MFM have been reported until now.[41 42] Many unknown causative genes may be involved in the pathogenesis of this disease. Therefore, it is increasingly important to accumulate genotype–phenotype spectrum of these relatively new rare diseases.

In conclusion, our genetic diagnosis technique combined with histological, mRNA and protein analyses is useful and efficient for screening pathogenic variants and for performing molecular diagnosis of patients with muscle diseases. The results of this study further emphasise the importance of developing a comprehensive disease mutation database and identifying multidimensional phenotypes from clinical, histological and molecular studies.

Web resources

- The URLs for data presented are as follows:
- Ensembl, http://www.ensembl.org/index.html
- OMIM, http://www.omim.org/
- 1000 Genomes, http://www.1000genomes.org/
- ESP6500, http://evs.gs.washington.edu/EVS/
- dbSNP138, http://www.ncbi.nlm.nih.gov/projects/SNP/
- HGVD, http://www.genome.med.kyoto-u.ac.jp/SnpDB/
- Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org/
- MutationTaster: http://www.mutationtaster.org
- Mutalyzer: http://www.mutalyzer.nl

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Patient consent Obtained.

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Targeted massively parallel sequencing and histological assessment of skeletal muscles for the molecular diagnosis of inherited muscle disorders

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Supplemental Table 1. MD panel

gene symbol	ENST#	NM#	Example of the phenotype	inheritance	OMIM
ACVR1	ENST00000263640	NM_001105.4	Fibrodysplasia ossificans progressiva	AD	MIM135100
AGRN	ENST00000379370	NM_198576.3	Myasthenic syndrome	AR	MIM615120
ALG13	ENST00000394780	NM_001099922.2	Congenital disorder of glycosylation	XLR	MIM300884
ANO5	ENST00000324559	NM_213599.2	LGMD2L	AR	MIM611307
B3GALNT2	ENST00000366600	NM_152490.2	alpha-dystroglycanopathy	AR	MIM615181
B3GNT1	ENST00000311181	NM_006876.2	alpha-dystroglycanopathy	AR	MIM615287
CAPN3	ENST00000397163	NM_000070.2	LGMD2A	AR	MIM253600
CAV3	ENST00000343849	NM_033337.2	LGMD1C	AD	MIM607801
CHKB	ENST00000406938	NM_005198.4	Megaconial myopathy	AR	MIM602541
COL12A1	ENST00000322507	NM_004370.5	Ullrich/Bethlem myopathy	AD/AR	MIM616470/616471
COL6A1	ENST00000361866	NM_001848.2	Ullrich/Bethlem myopathy	AD/AR	MIM254090/158810
COL6A2	ENST00000300527	NM_001849.3	Ullrich/Bethlem myopathy	AD/AR	MIM254090/158810
COL6A3	ENST00000295550	NM_004369.3	Ullrich/Bethlem myopathy	AD/AR	MIM254090/158810
DAG1	ENST00000545947	NM_001177634.2	Dystroglycanopathy	AR	MIM616538
DES	ENST00000373960	NM_001927.3	Myofibrillar myopathy/LGMD2R	AD/AR	MIM601419/615325
DMD	ENST00000357033	NM_004006.2	Duchenne/Becker Muscular Dystrophy	XLR	MIM310200/300376
DNAJB6	ENST00000262177	NM_058246.3	LGMD1E	AD	MIM603511
DOK7	ENST00000389653	NM_173660.4	Myasthenia, limb girdle	AR	MIM254300
DOLK	ENST00000372586	NM_014908.3	Congenital disorder of glycosylation	AR	MIM610768
DPAGT1	ENST00000409993	NM_001382.3	Congenital disorder of glycosylation/Myathenic syndrome	AR	MIM608093/614750
DPM1	ENST00000371584	NM_003859.1	Congenital disorder of glycosylation	AR	MIM608799
DPM2	ENST00000373110	NM_003863.3	Congenital disorder of glycosylation	AR	MIM615042
DPM3	ENST00000368399	NM_018973.3	Congenital disorder of glycosylation	AR	MIM612937
DYSF	ENST00000258104	NM_003494.3	LGMD2B	AR	MIM253601
EMD	ENST00000369842	NM_000117.2	Emery-Dreifuss muscular dystrophy	XLR	MIM310300
FAT1	ENST00000441802	NM_005245.3	Facioscapulohumeral muscular dystrophy-like myopathy?	AD	
FHL1	ENST00000394155	NM_001159702.2	Reducing body myopathy	XLD	MIM300717
FKRP	ENST00000318584	NM_024301.4	alpha-dystroglycanopathy	AR	MIM613153
FKTN	ENST00000602661	NM_001079802.1	alpha-dystroglycanopathy	AR	MIM253800
FLNC	ENST00000325888	NM_001458.4	MFM	AD/AR	MIM609524
GFPT1	ENST00000357308	NM_001244710.1	Congenital myathenia	AR	MIM610542
GMPPB	ENST00000308375		alpha-dystroglycanopathy	AR	MIM615350
ISPD	ENST00000407010	NM_001101426.3	alpha-dystroglycanopathy	AR	MIM614643
ITGA7	ENST00000257880	XM_005268841.1	Congenital muscular dystrophy	AR	MIM613204
KLHL9	ENST00000359039	NM_018847.2	Distal myopathy?	AD	
LAMA2	ENST00000421865	NM_000426.3	Congenital muscular dystrophy	AR	MIM607855
LARGE	ENST00000354992	NM_004737.4	alpha-dystroglycanopathy	AR	MIM613154
LMNA	ENST00000368300	NM_170707.3	LGMD1B	AD	MIM159001
MEGF10	ENST00000274473	NM_032446.2	EMARDD	AR	MIM614399
MICU1	ENST00000398761	NM_006077.3	Myopathy with extrapyramidal signs	AR	MIM615673
MYOT	ENST00000239926	NM_006790.2	LGMD1A/MFM	AD	MIM609200
PLEC1	ENST00000322810	NM_201380.3	Muscular dystrophy with epidermolysis bullosa simplex/LGMD2Q	AR	MIM226670
POMGNT1	ENST00000371992	NM_001243766.1	alpha-dystroglycanopathy	AR	MIM253280
POMGNT2	ENST00000344697	NM_032806.5	alpha-dystroglycanopathy	AR	MIM614830
POMT1	ENST00000372228	NM_007171.3	alpha-dystroglycanopathy	AR	MIM236670
POMT2	ENST00000261534	NM_013382.5	alpha-dystroglycanopathy	AR	MIM613150
PTRF	ENST00000357037	NM_012232.5	Muscular dystrphy and lipodystrophy	AR	MIM613327
SGCA	ENST00000262018	NM_000023.2	LGMD2D	AR	MIM608099
SGCB	ENST00000381431	NM_000232.4	LGMD2E	AR	MIM604286
SGCD	ENST00000337851	NM_000337.5	LGMD2F	AR	MIM601287
SGCG	ENST00000218867	NM_000231.2	LGMD2C	AR	MIM253700
POMK	ENST00000331373	NM_032237	alpha-dystroglycanopathy	AR	MIM615249
SMCHD1	ENST00000320876	NM_015295.2	Facioscapulohumeral muscular dystrophy type2	AD	MIM158901
STIM1	ENST00000300737	NM_003156.3	Tubular aggregate myopathy	AD	MIM160565
SYNE1	ENST00000367255	NM_182961.3	Emery-Dreifuss muscular dystrophy	AD	MIM612998
SYNE2	ENST00000358025	NM_182914.2	Emery-Dreifuss muscular dystrophy	AD	MIM612999
TCAP	ENST00000309889	NM_003673.3	LGMD2G	AR	MIM601954
TMEM43	ENST00000306077	NM_024334.2	Emery-Dreifuss muscular dystrophy?	AD	MIM614302
TMEM5	ENST00000261234	NM_014254.2	alpha-dystroglycanopathy	AR	MIM615041
TNPO3	ENST00000393245	NM_012470.3	LGMD1F	AD	MIM608423
TRAPPC11	ENST00000334690	NM_021942.5	LGMD2S	AR	MIM615356
TRIM32	ENST00000450136	NM_012210.3	LGMD2H	AR	MIM254110

Supplemental Table 2. CMP panel

gene symbol	ENST#	NM#	Example of the phenotype	inheritance	OMIM
ACTA1	ENST00000366684	NM_001100.3	Nemaline myopathy	AD/AR	MIM161800
AGRN	ENST00000379370	NM_198576.3	Myathenic syndrome	AR	MIM103320
ALG14	ENST00000370205	NM_144988.3	Myathenic syndrome	AR	MIM612866
ALG2	ENST00000476832	NM_033087.3	Myathenic syndrome	AR	MIM607905
BIN1	ENST00000316724	NM_139343.2	Centronuclear myopathy	AR	MIM255200
CCDC78	ENST00000293889	NM_001031737.2	Centronuclear myopathy	AD	MIM614807
CFL2	ENST00000341223	NM_021914.7	Nemaline myopathy	AR	MIM610687
CHAT	ENST00000337653	NM_020549.4	Myathenic syndrome	AR	MIM118491
CHRNA1	ENST00000261007	NM_001039523.2	Myathenic syndrome	AD/AR	MIM100690
CHRNB1	ENST00000306071	NM_000747.2	Myathenic syndrome	AD/AR	MIM100710
CHRND	ENST00000258385	NM_000751.2	Myathenic syndrome	AD/AR	MIM100720
CHRNE	ENST00000293780	NM_000080.3	Myathenic syndrome	AD/AR	MIM100725
CHRNG	ENST00000389494	NM_005199.4	Escobar syndrome	AR	MIM265000
CNTN1	ENST00000551295	NM_001843.3	CMP	AR	MIM612540
COLQ	ENST00000383788	NM_005677.3	Myathenic syndrome	AR	MIM603033
DNM2	ENST00000355667	NM_001005360.2	Centronuclear myopathy	AD	MIM160150
DOK7	ENST00000389653	NM_173660.4	Myathenic syndrome	AR	MIM610285
DPAGT1	ENST00000409993	NM_001382.3	Congenital disorder of glycosylation/Myathenic syndrome	AR	MIM191350
GFPT1	ENST00000361060	NM_002056.3	Congenital myathenia	AR	MIM138292
HSPG2	ENST00000374695	NM_005529.6	Schwartz-Jampel syndrome	AR	MIM255800
KBTBD13	ENST00000432196	NM_001101362.2	Nemaline myopathy	AD	MIM609273
KLHL40	ENST00000287777	NM_152393.3	Nemaline myopathy	AR	MIM615348
LAMB2	ENST00000418109	NM_002292.3	Myathenic syndrome/Piearson synd	AR	MIM150325
LRP4	ENST00000378623	NM_002334.3	Myathenic syndrome?	AR	MIM616304
MEGF10	ENST00000274473	NM_032446.2	EMARDD	AR	MIM614399
MTM1	ENST00000370396	NM_000252.2	Myotubular myopathy	XLR	MIM310400
MUSK	ENST00000189978	NM_005592.3	Myathenic syndrome	AR	MIM 601296
MYBPC3	ENST00000545968	NM_000256.3	Cardiomyopathy/Myopathy	AD/AR	MIM615396
MYH7	ENST00000355349	XM_005267696.1	Distal myopathy/Myosin storage myopathy/Dilated cardiomyopathy	AD	MIM160500
NEB	ENST 00000427231	NM_1271208.1	Nemaline myopathy	AR	MIM256030
ORAI1	ENST00000330079	NM_032790.3	Tubular aggregate myopathy	AD	MIM615883
PLEC	ENST00000322810	NM_201380.3	Muscular dystrophy with epidermolysis bullosa simplex	AR	MIM 601282
PTPLA	ENST00000361271	NM_014241.3	Centronuclear myopathy?	AR	—
RAPSN	ENST00000524487	NM_005055.4	Myathenic syndrome	AR	MIM 601592
RYR1	ENST00000359596	NM_000540.2	Malignant hyperthermia/Central core disease	AD/AR	145600/117000
SCN4A	ENST00000578147	NM_000334.4	Paramyotonia congenita/Myathenic syndrome/	AD/AR	MIM168300/614198
SEPN1	ENST00000361547	NM_020451.2	Multiminicore disease	AR	MIM602771
STIM1	ENST00000300737	NM_003156.3	Tubular aggregate myopathy	AD	MIM160565
TNNT1	ENST00000588981	NM_003283.5	Nemaline myopathy	AR	MIM605355
TPM2	ENST00000378300	NM_003289.3	Nemaline myopathy	AD/AR	MIM609285
TPM3	ENST00000368530	NM_152263.2	Nemaline myopathy	AD	MIM609284

Supplemental Table 3. MM panel

gene symbol	ENST#	NM#	Example of the phenotype	inheritance	
ABHD5	ENST00000458276	NM_016006.4	Chanarin-Dorfman syndrome	AR	MIM275630
ACADM	ENST00000370834	NM_000016.4	Acyl-CoA dehydrogenase, medium chain, deficiency of	AR	MIM201450
ACADS	ENST00000242592	NM_000017.2	Acyl-CoA dehydrogenase, short-chain, deficiency of	AR	MIM201470
ACADVL	ENST00000356839	NM_000018.3	VLCAD deficiency	AR	MIM201475
AGL	ENST00000361915	NM_000642.2	Glycogen storage disease IIIa, b	AR	MIM232400
ALDOA	ENST00000395248	NM_000034.3	Glycogen storage disease XII	AR	MIM611881
CACNA1S	ENST00000362061	NM_000069.2	Hypokalemic periodic paralysis/Malignant hyperthermia susceptibility	AD	MIM170400
CLCN1	ENST00000343257	NM_000083.2	Myotonia congenita	AD/AR	MIM160800/255700
CPT2	ENST00000371486	NM_000098.2	Myopathy due to CPT II deficiency	AR	MIM255110
ENO3	ENST00000323997	NM_001976.4	Glycogen storage disease XIII	AR	MIM612932
ETFA	ENST00000557943	NM_000126.3	Glutaric acidemia IIA	AR	MIM231680
ETFB	ENST00000354232	NM_001985.2	Glutaric acidemia IIB	AR	MIM231680
ETFDH	ENST00000511912	NM_004453.2	Glutaric acidemia IIC	AR	MIM231680
GAA	ENST00000302262	NM_000152.3	Glycogen storage disease II	AR	MIM232300
GBE1	ENST00000429644	NM_000158.3	Glycogen storage disease IV/Polyglucosan body disease	AR	MIM232500/263570
GYG1	ENST00000345003	NM_004130.3	Glycogen storage disease XV/Polyglucosan body myopathy 2	AR	MIM613507/616199
GYS1	ENST00000323798	NM_002103.4	Glycogen storage disease 0	AR	MIM611556
HADHA	ENST00000380649	NM_000182.4	Trifunctional protein deficiency	AR	MIM609015
HADHB	ENST00000317799	NM_000183.2	Trifunctional protein deficiency	AR	MIM609015
ISCU	ENST00000311893	NM_213595.3	Myopathy with lactic acidosis	AR	MIM255125
LDHA	ENST00000540430	NM_001165414.1	Glycogen storage disease XI	AR	MIM612933
LPIN1	ENST00000449576	NM_145693.2	Myoglobinuria	AR	MIM268200
MICU1	ENST00000398761	NM_006077.3	Myopathy with extrapyramidal signs	AR	MIM615673
MTO1	ENST00000415954	NM_001123226.1	Combined oxidative phosphorylation deficiency 10	AR	MIM614702
PFKM	ENST00000340802	NM_001166686.1	Glycogen storage disease VII	AR	MIM232800
PGAM2	ENST00000297283	NM_000290.3	Glycogen storage disease X	AR	MIM261670
PGK1	ENST00000373316	NM_000291.3	Phosphoglycerate kinase 1 deficiency	XLR	MIM300653
PGM1	ENST00000371083	NM_001172818.1	Congenital disorder of glycosylation, type XIV	AR	MIM614921
PHKA1	ENST00000373539	NM_002637.3	Glycogen storage disease IXd	XLR	MIM300559
PNPLA2	ENST00000336615	NM_020376.3	Neutral lipid storage disease with myopathy	AR	MIM610717
PRKAG2	ENST00000287878	NM_016203.3	Glycogen storage disease of heart, lethal congenital	AD	MIM261740
PYGM	ENST00000164139	NM_005609.2	McArdle disease/Glycogen storage disease V	AR	MIM232600
SCN4A	ENST00000578147	NM_000334.4	Paramyotonia congenita	AD	MIM168300
SLC22A5	ENST00000435065	NM_003060.3	Primary systemic carnitine deficiency	AR	MIM212140
SLC25A20	ENST00000319017	NM_000387.5	Carnitine/acyl- carnitine translocase deficiency	AR	MIM212138
TAZ	ENST00000299328	NM_000116.3	Barth syndrome	XLR	MIM302060

Supplemental Table 4. MFM panel

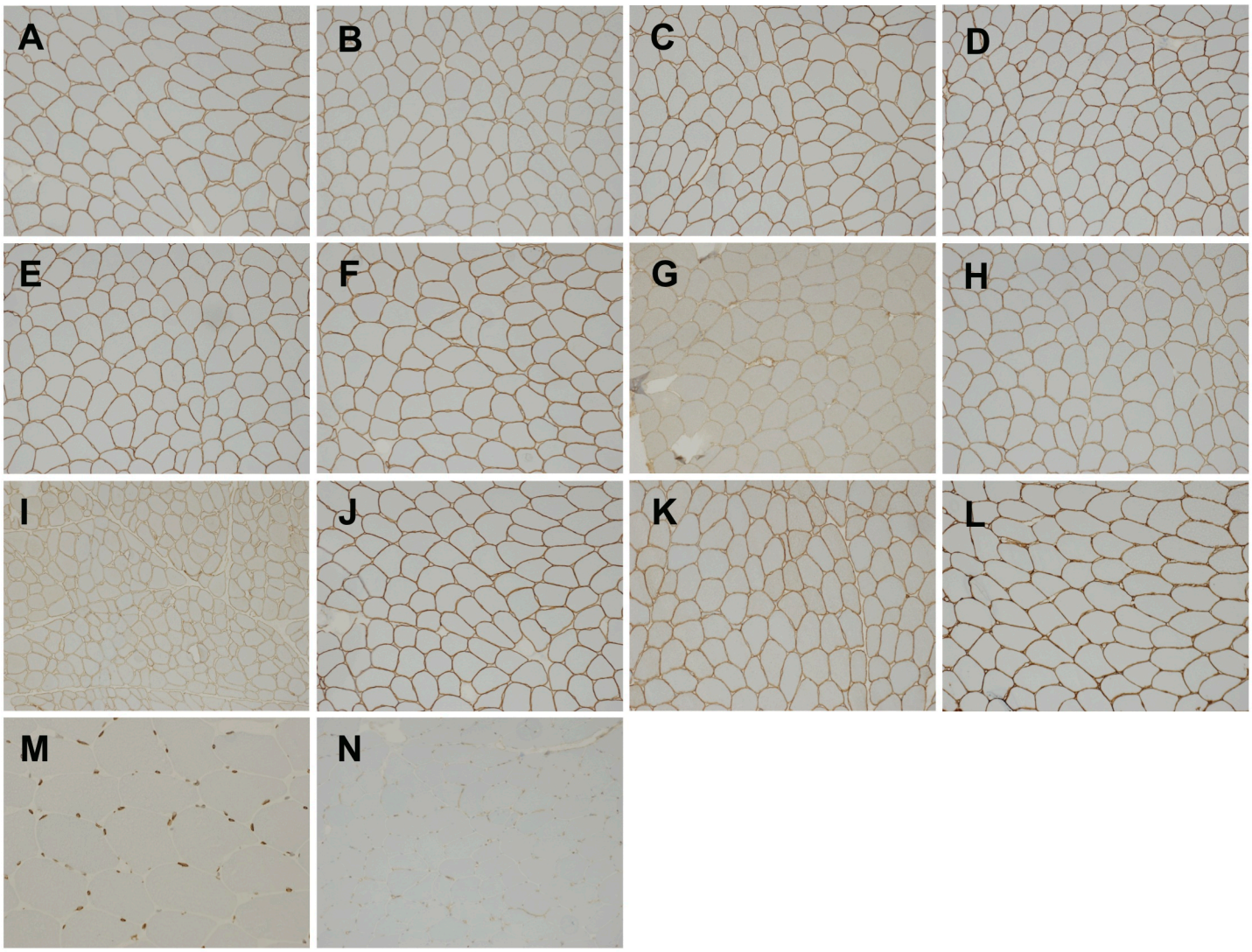
gene symbol	ENST#	NM#	Example of the phenotype	inheritance	OMIM
ACTA1	ENST00000366684	NM_001100.3	Nemaline myopathy	AD/AR	MIM161800
BAG3	ENST00000369085	NM_004281.3	MFM	AD	MIM612954
CFL2	ENST00000341223	NM_021914.7	Nemaline myopathy	AR	MIM610687
CRYAB	ENST00000533475	NM_001885.1	MFM	AD/AR	MIM608810/613869
DES	ENST00000373960	NM_001927.3	MFM	AD	MIM601419
DNAJB6	ENST00000262177	NM_058246.3	LGMD1E/MFM	AD	MIM603511
EPG5	ENST00000282041	NM_020964.2	Vici syndrome	AR	MIM242840
FHL1	ENST00000394155	NM_001159702.2	Reducing body myopathy	XLD	MIM300717
FLNC	ENST00000325888	NM_001458.4	MFM	AD	MIM609524
GNE	ENST00000396594	NM_001128227.2	DMRV	AR	MIM605820
KBTBD13	ENST00000432196	NM_001101362.2	Nemaline myopathy	AD	MIM609273
KLHL40	ENST00000287777	NM_152393.3	Nemaline myopathy	AR	MIM615348
LAMP2	ENST00000371335	NM_013995.2	Danon disease	XLD	MIM300257
LDB3	ENST00000429277	NM_001171610.1	MFM	AD	MIM609452
MATR3	ENST00000394800	NM_199189.2	ALS/VCPDM	AD	MIM606070
MEGF10	ENST00000274473	NM_032446.2	EMARDD	AR	MIM614399
MYH2	ENST00000245503	NM_017534.5	Proximal myopathy and ophthalmoplegia	AD/AR	MIM605637
MYH7	ENST00000355349	XM_005267696.1 ?	Distal myopathy/Myosin storage myopathy/Dilated cardiomyopathy	AD	MIM160500
MYOT	ENST00000239926	NM_006790.2	LGMD1A/MFM	AD	MIM609200
NEB	ENST00000397345	NM_001164508.1	Nemaline myopathy	AR	MIM256030
ORAI1	ENST00000330079	NM_032790.3	Tubular aggregate myopathy	AD	MIM615883
PABPN1	ENST00000216727	NM_004643.3	Oculopharyngeal muscular dystrophy	AD	MIM164300
PLEC	ENST00000322810	NM_201380.3	Muscular dystrophy with epidermolysis bullosa simplex	AR	MIM226670
RBCK1	ENST00000356286	NM_031229.2	Polyglucosan body myopathy	AR	MIM615895
SEPN1	ENST00000361547	NM_020451.2	Multiminicore disease	AR	MIM602771
SIL1	ENST00000394817	NM_022464.4	Marinesco-Sjogren syndrome	AR	MIM248800
STIM1	ENST00000300737	NM_003156.3	Tubular aggregate myopathy	AD	MIM160565
TCAP	ENST00000309889	NM_003673.3	LGMD2G	AR	MIM601954
TIA1	ENST00000433529	NM_022173.2	Welander distal myopathy	AD	MIM604454
TNNT1	ENST00000588981	NM_003283.5	Nemaline myopathy	AR	MIM605355
TPM2	ENST00000378300	NM_003289.3	Nemaline myopathy	AD	MIM609285
TPM3	ENST00000368530	NM_152263.2	Nemaline myopathy	AD	MIM609284
TRIM32	ENST00000373983	NM_001099679.1	LGMD2H	AR	MIM254110
TTN	ENST00000589042	NM_001267550.2	Tibial muscular dystrophy/HMERF/LGMD2J	AD/AR	MIM600334/603689/608807
VCP	ENST00000358901	NM_007126.3	IBMPFD	AD	MIM167320
VMA21	ENST00000330374	NM_001017980.3	X-linked myopathy with excessive autophagy	XLR	MIM310440

Supplemental Table 5

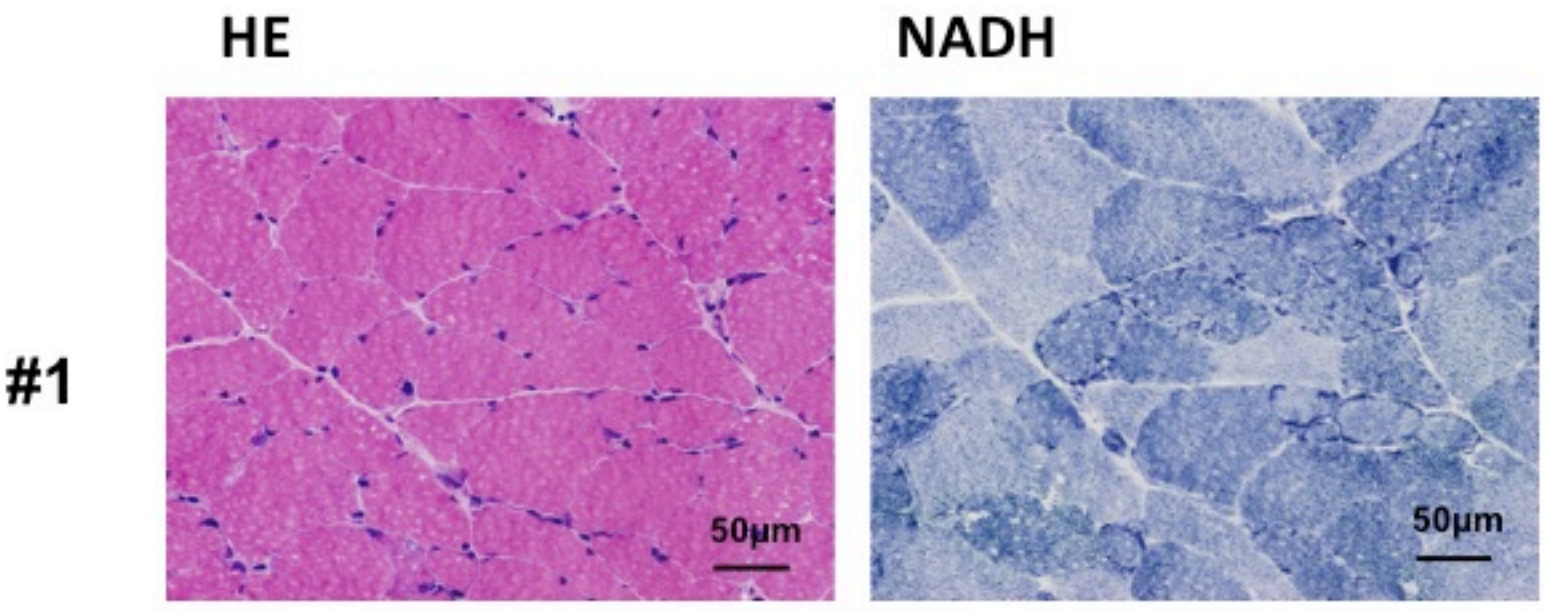
#	Age	sex	Ethnicity	phenotype	gene	cDNA	status	protein	1000g	ESP6500	HGVD	ExAC	Clin var	Suppl. Fig.	Mutation Taster	Reported
63	6y	M	J	EDMD	*LMNA*	c.107A>T	het	p.(Gln36Leu)	–	–	–	–	–		disease causing	no
64	22y	M	J	LGMD	*LMNA*	c.1095C>G	het	p.(Ile365Met)	–	–	–	–	–		disease causing	no
65	48y	M	J	CNM	*DNM2*	c.1871G>T	het	p.(Gly624Val)	–	–	–	–	–	35	disease causing	no
66	62y	M	J	CNM	*DNM2*	c.1483G>A	het	p.(Gly495Arg)	–	–	–	0.000008	–	35	disease causing	no
67	2y	F	J	Nemaline myopathy	*NEB*	c.24282_24285dup	het	p.(Glu8096Serfs*5)	–	–	–	–	–	36	disease causing	no
					NEB	c.22924del	het	p.(Tyr7642Metfs*10)	–	–	–	–	–		disease causing	no
					NEB	c.17606C>T	het	p.(Ala5869Val)	0.0018	–	0.0063		–		disease causing	no
68	63y	F	J	Nemaline myopathy	*NEB*	c.24275A>G	het	p.(Lys8092Arg)	–	–	0.0054	0.000191	–	36	disease causing	no
					NEB	c.9713A>T	het	p.(Asn3238Ile)	0.0005	–	0.0023	0.000015	–		disease causing	no
69	63y	M	J	Nemaline myopathy	*NEB*	c.22924del	het	p.(Tyr7642Metfs*10)	–	–	–	–	–	36	disease causing	no
						c.20131C>T	het	p.(Arg6711Trp)	–	–	0.0021	0.000050	–		disease causing	no
70	13y6m	M	J	Nemaline myopathy	*NEB*	c.C20131T	het	p.(Arg6711Trp)	–	–	0.0021	0.000050	–	36	disease causing	no
					NEB	c.7755delT	het	p.(Ser2585fs)	–	–	–	–	–		disease causing	no
71	37y	M	J	Nemaline myopathy	*NEB*	c.20131C>T	het	p.(Arg6711Trp)				0.000050	–	36	disease causing	no
					NEB	c.9046C>T	het	p.(Arg3016*)				–	–		disease causing	yes
72	1y	F	J	CMP_uniform type1	*RYR1*	c.7487C>T	het	p.(Pro2496Leu)	–	–	0.0014	0.000083	Uncertain significance	37	disease causing	yes
					RYR1	c.14560G>A	het	p.(Val4854Met)	–	–	–	–	–		disease causing	no
73	8m	M	J	CFTD	*RYR1*	c.5861G>A	het	p.(Arg1954His)	–	–	–	0.000008	–	38,39	disease causing	no
					RYR1	c.9472+1G>A	het		–	–	–	0.000008	–		disease causing	no
					RYR1	c.10664A>T	het	p.(Asn3555Ile)	–	–	0.0027	0.000099	Uncertain significance		disease causing	no
74	6m	F	J	CFTD	*RYR1*	c.497delA	het	p.(Asp166Valfs*36)	–	–	–	–	–	38	disease causing	no
					RYR1	c.5861G>A	het	p.(Arg1954His)	–	–	–	0.000008	–		disease causing	no
					RYR1	c.10664A>T	het	p.(Asn3555Ile)	–	–	0.0027	0.000099	Uncertain significance		disease causing	no
75	1y3m	F	J	CFTD	*RYR1*	c.7836-1G>A	het		–	–	–	–	–	40,41	disease causing	no
					RYR1	c.13673G>A	het	p.(Arg4558Gln)	0.0005	0.000077	–	0.000016	not provided		disease causing	yes
76	3y10m	F	J	Type 1 fiber predominance	*RYR1*	c.12083C>T	het	p.(Ser4028Leu)	–	–	–	–	Uncertain significance	42	disease causing	yes
77	0y	M	J	Type 1 fiber predominance	*RYR1*	c.14438A>G	het	p.(His4813Arg)	–	–	–	–	–	42	disease causing	yes

Supplemental Table 6.

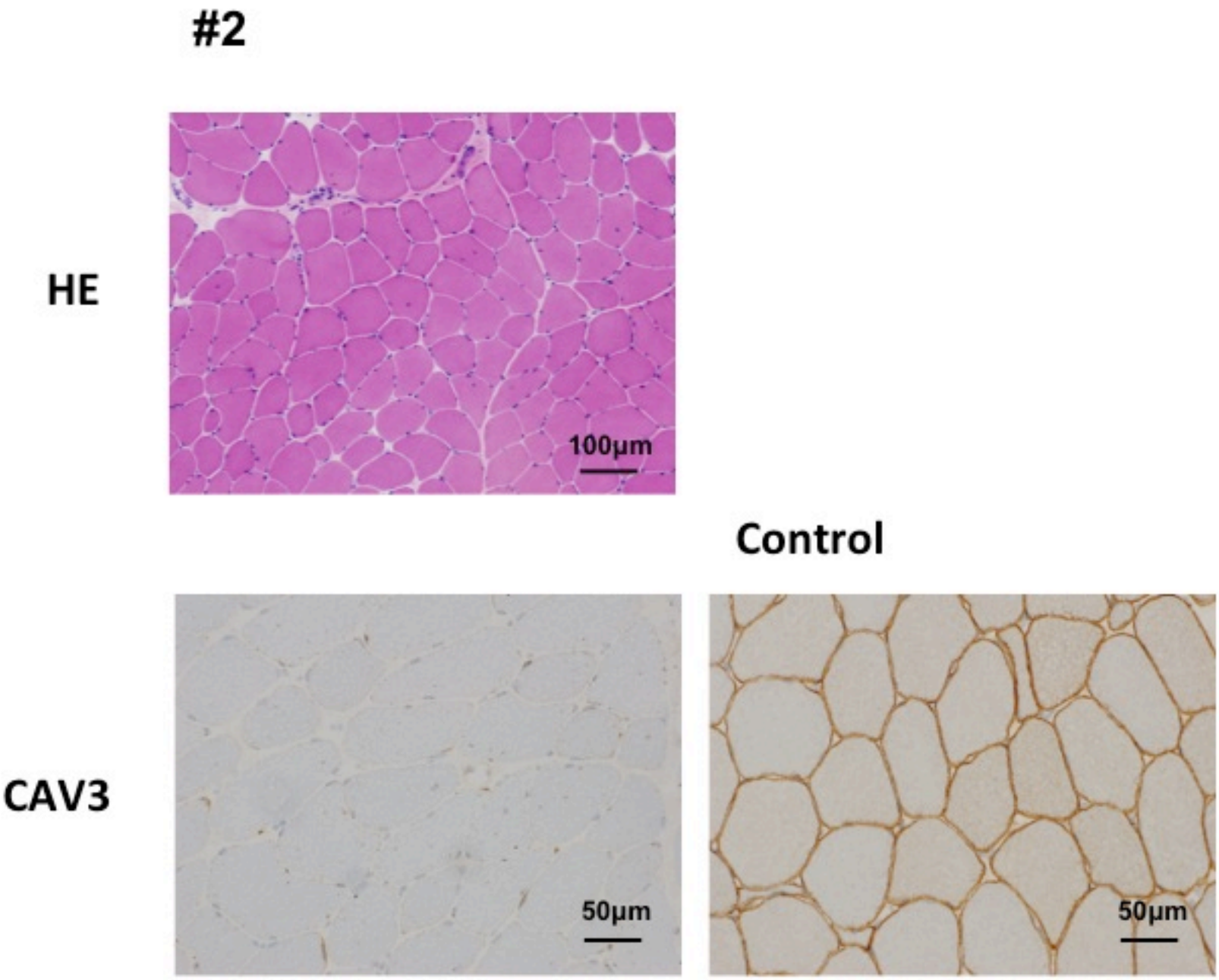
SGCB_cDNA_Ex3_Fw	CACAGTAGGAGGAAGGCGAA
SGCB_cDNA_Ex6_Rv	CCAGTCACCACTACCCAACT
MTM1_cDNA_Ex11Fw	TGCTTGTGCATTGCAGTGAC
MTM1_cDNA_Ex14-15Rv	CTCCACTGGATTCGGCTGTT
AGL_cDNA_Ex10_Fw	AGGACCTGTCACTAGAAAGCA
AGL_cDNA_Ex14_Rv	GCCTCAAACAGGGCTGAACA
RYR1_cDNA_Ex48_Fw	CGCCATCATGGTGGACTCTA
RYR1_cDNA_Ex52_Rv	TCGTGTGTGTACTCCGCAAA
COL6A3_cDNA_Ex16_Fw	TCCTGGAGAAGACGGCTACC
COL6A3_cDNA_Ex23-24_Rv	GCCAAAGCCACCATTCTTCC
COL6A2_cDNA_Ex3-1_Fw	CCTGCACTTCTCTGACCAGG
COL6A2_cDNA_Ex7_Rv	GAATCCAATGGGGCCTTCGA
COL6A2_cDNA_Ex6_Fw	CTGGCCAGAAGGGAAGACAG
COL6A2_cDNA_Ex14_Rv	GCCCTTGGCTCCTTTCACA
RYR1_cDNA_Ex63_Fw	GAAGTCAGGCCCTGAGATCG
RYR1_cDNA_Ex65_RV	CGTTGTACTCGTTCAGCTGC



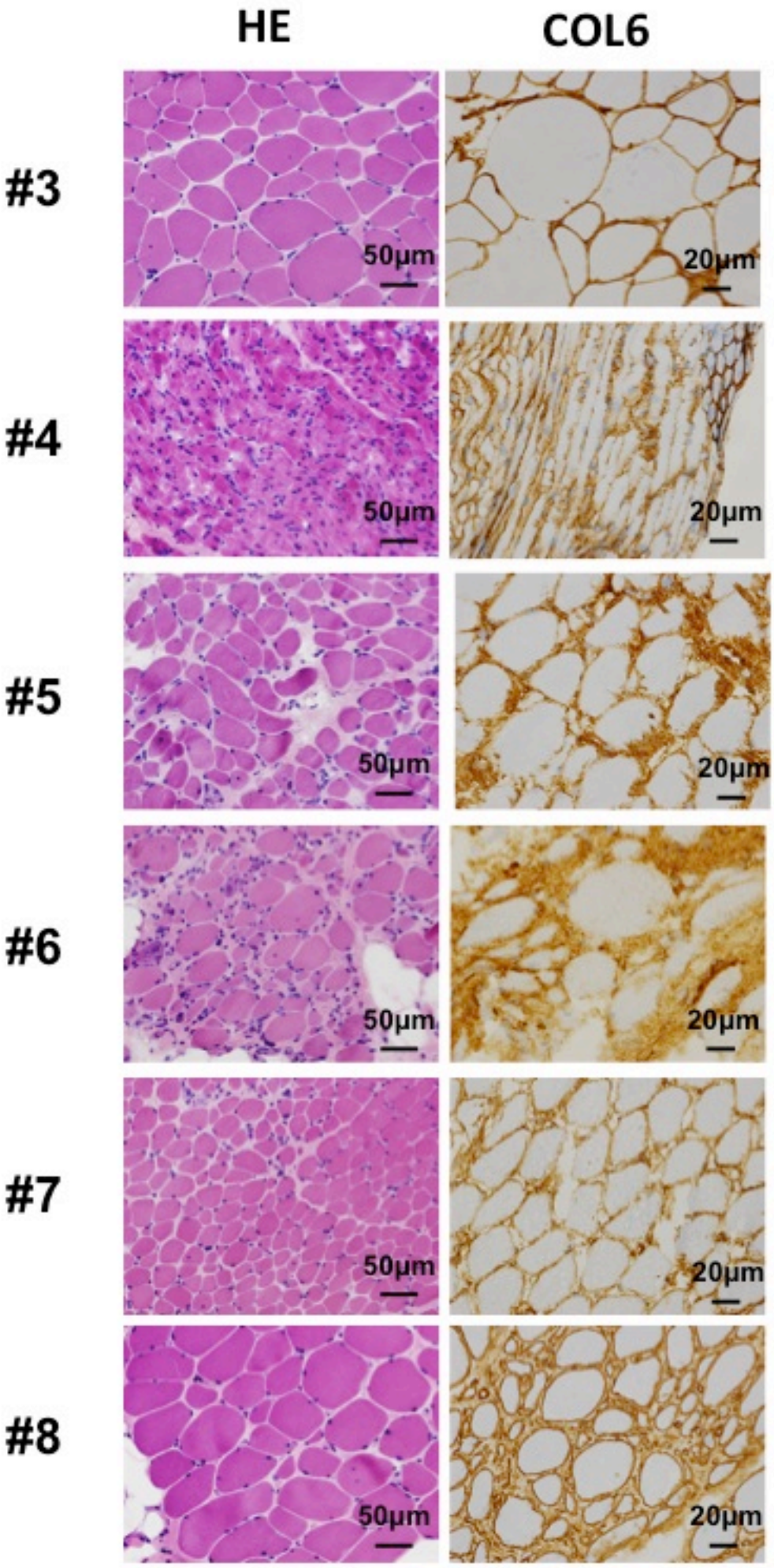
Supplemental Figure 1
Immunohistochemical analysis of muscular dystrophy proteins: dystrophin rod (DYS1) (A), dystrophin C-terminal (DYS2) (B), dystrophin N-terminal (DYS3) (C), α-sarcoglycan (D), β-sarcoglycan (E), γ-sarcoglycan (F), glycosylated α-dystroglycan (G), β-dystroglycan (H), dysferlin (I), merosin M-chain (J), caveolin3 (K), collagen type VI (L), emerin (M) and utrophin (N).



Supplemental Figure 2
Histochemical staining of biopsied muscles from patient with homozygous *CAPN3* mutation R461C (#1). HE staining showed nonspecific mild variation in fiber size. On NADH staining, typical moth-eaten fibers are seen, suggesting calpainopathy.



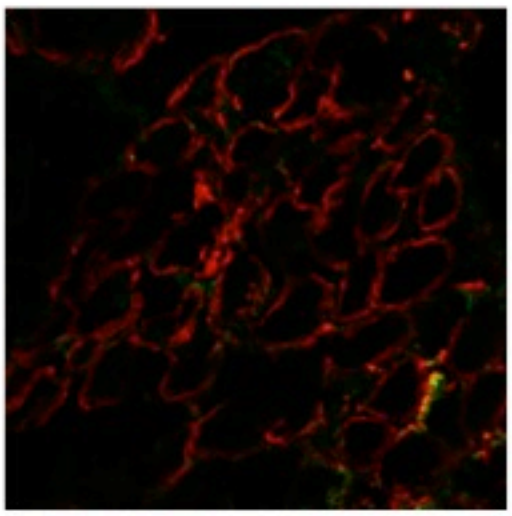
Supplemental Figure 3
HE staining showed nonspecific changes with variation in fiber size in a patient with a *CAV3* mutation (#2). Caveolin3 was absent in the patient's muscle, indicative of caveolinopathy.



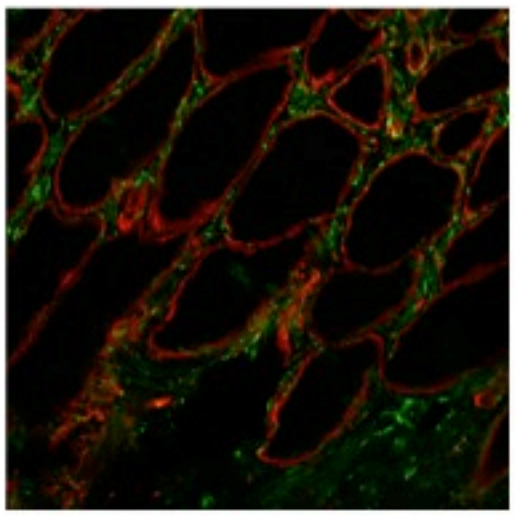
Supplemental Figure 4
All patients with *COL6A1* (#3, 4), *COL6A2* (#5, 6) and *COL6A3* (#7, 8) mutations showed moderate endomysial fibrosis with various levels of dystrophic changes. Immunohistochemical staining of collagen type VI showed sarcolemma-specific collagen deficiency (SSCD) in patients # 3, 4, 5, 6 and 7. In patient # 8, the collagen type VI defect was not obvious.

merge

#3



#4



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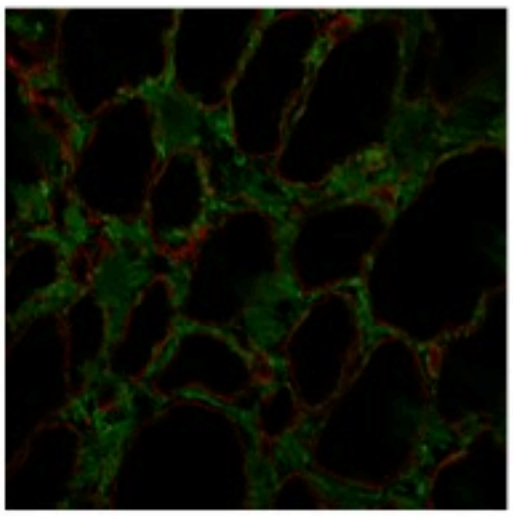

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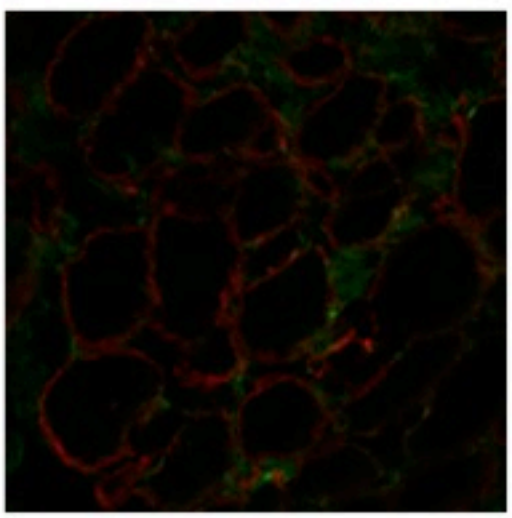

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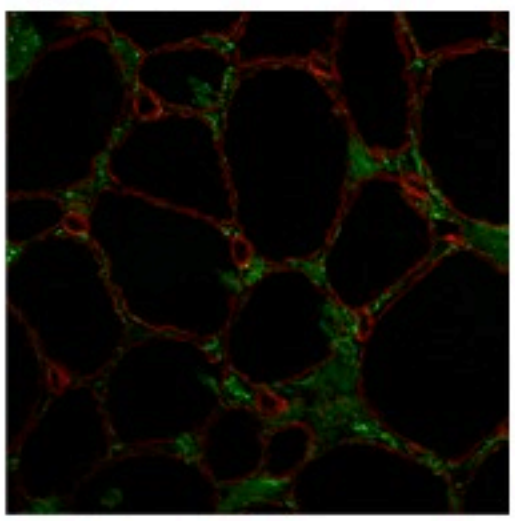
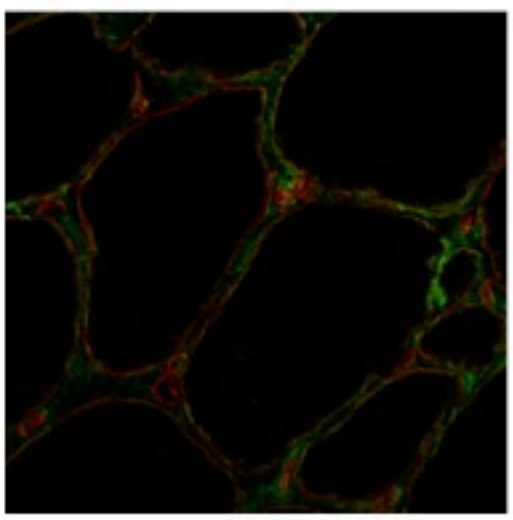
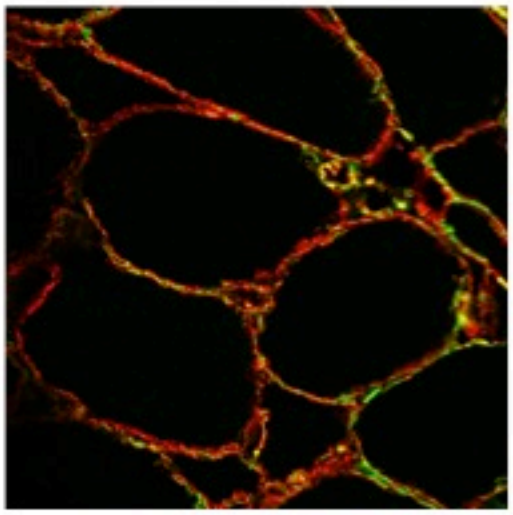


#8



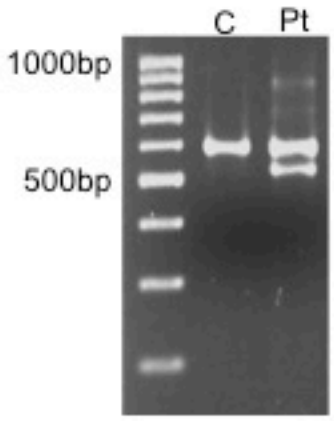
control



Supplemental Figure 5
Immunofluorescent staining of collagen type IV (red) and collagen type VI (green). All patients with *COL6A1*, *COL6A2* and *COL6A3* mutations showed sarcolemma specific collagen deficiency (SSCD). Merged images are shown.

#5

A



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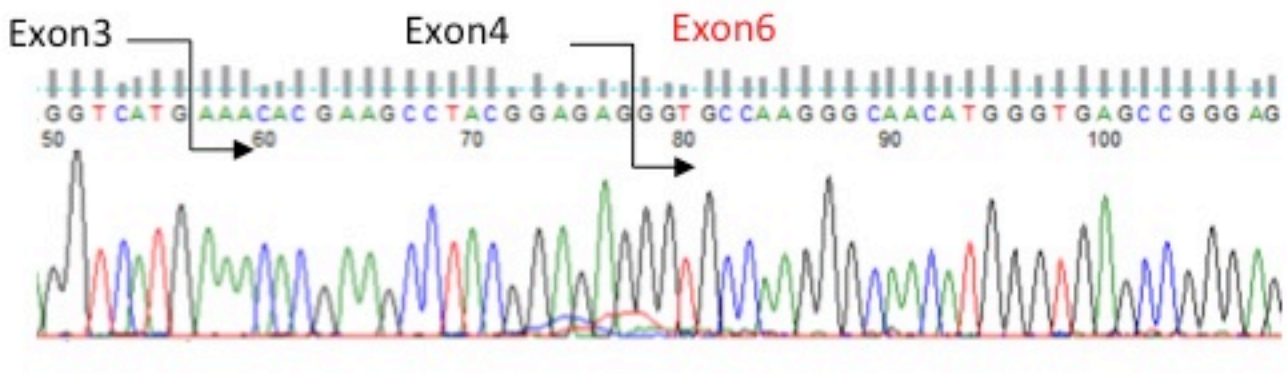

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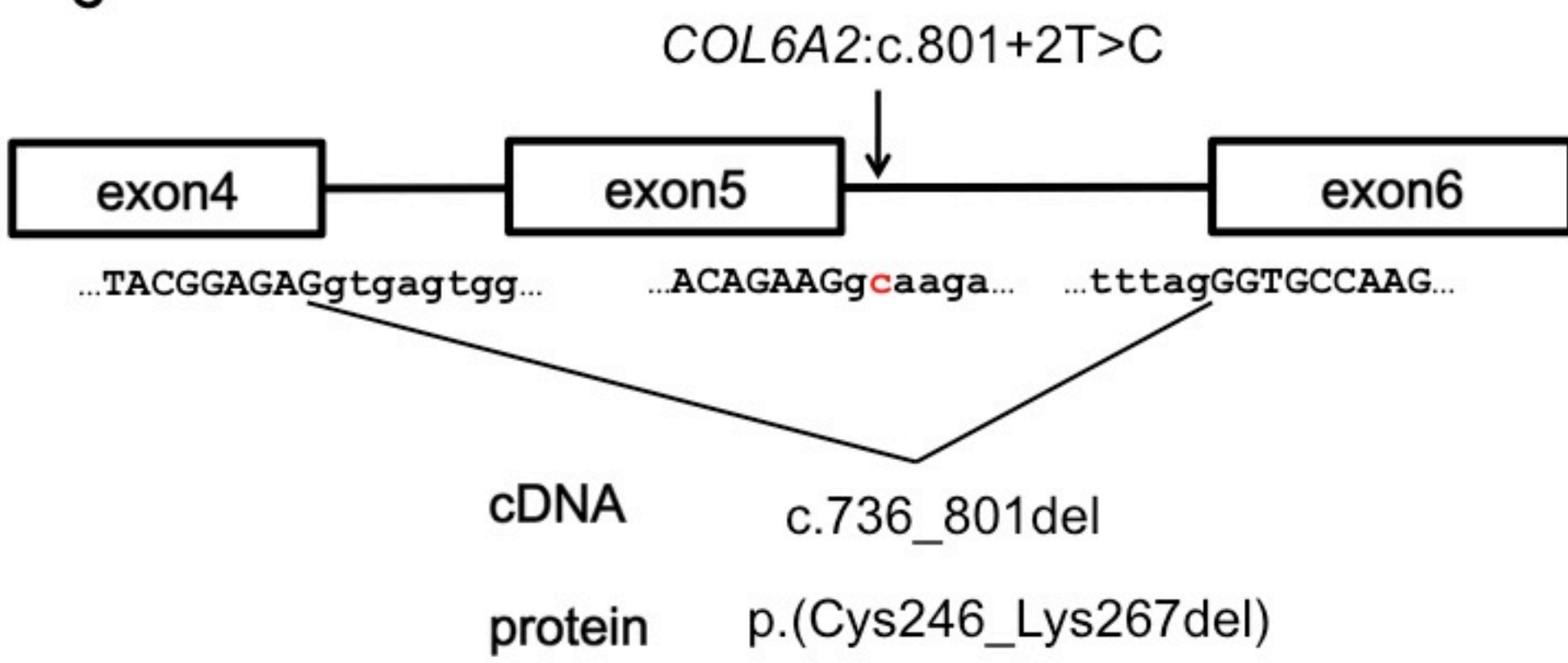
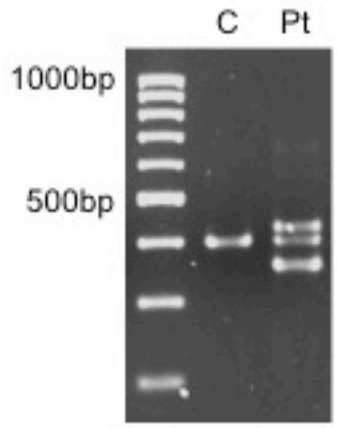


Supplemental Figure 6
Patient with splice site mutation in *COL6A2* (#5). (A-B) Muscle cDNA analysis using primers in exon 3 and 7 showed that normal transcript and aberrant spliced transcript, which skips exon 5, are expressed. C: control. Pt: patient. (C) Exon 5 skipping results in 22-amino acid in-frame deletion. This 22-amino acid deletion disrupts the triple-helical region, which is responsible for dominant Ullrich muscular dystrophy.

#6

A



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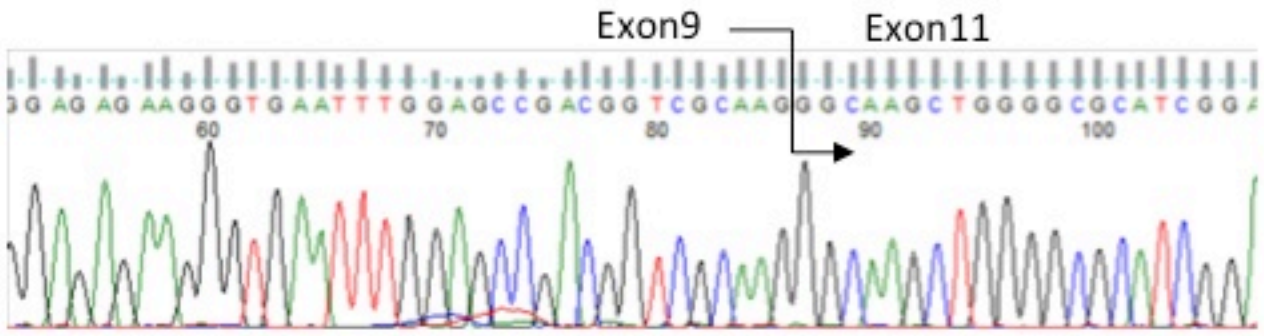
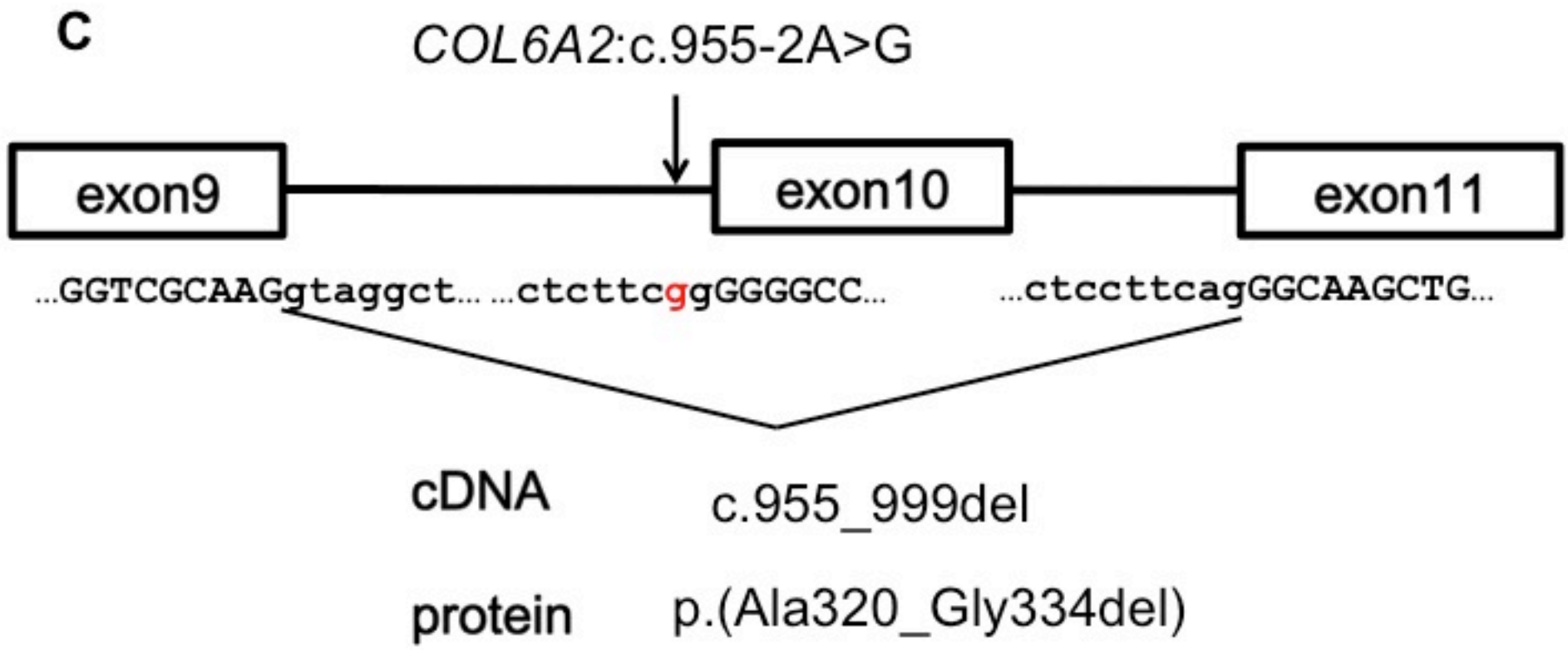
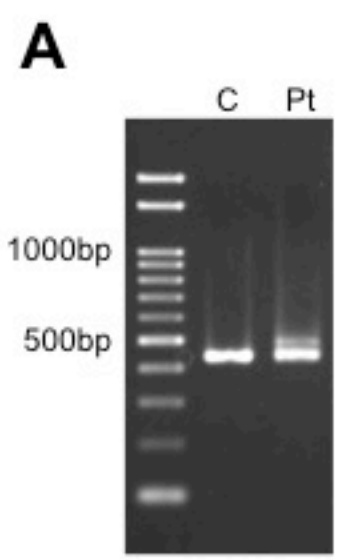


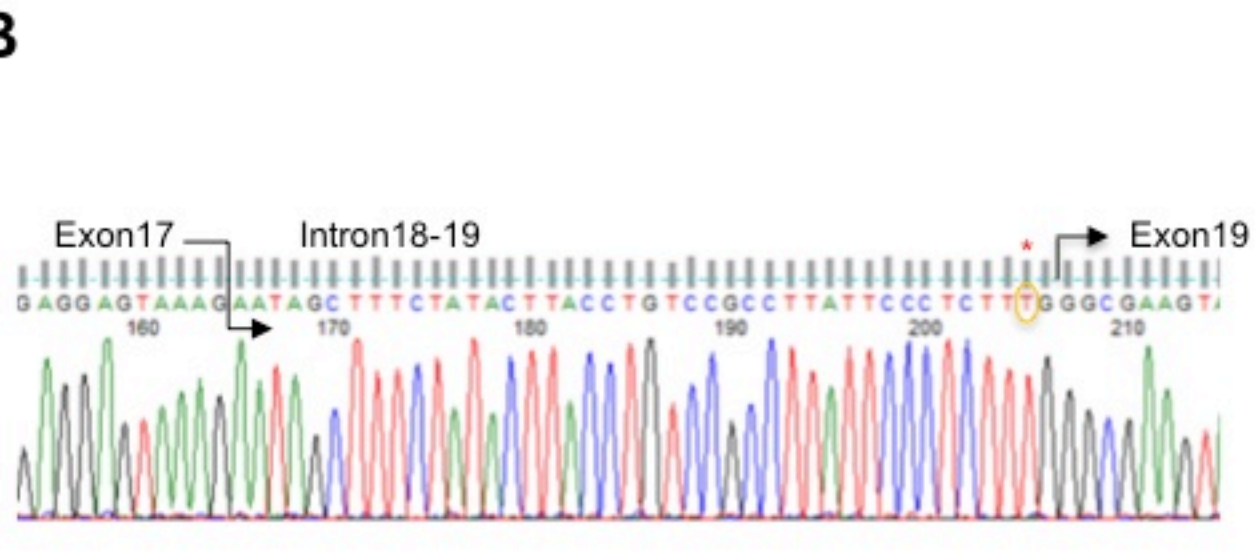
C

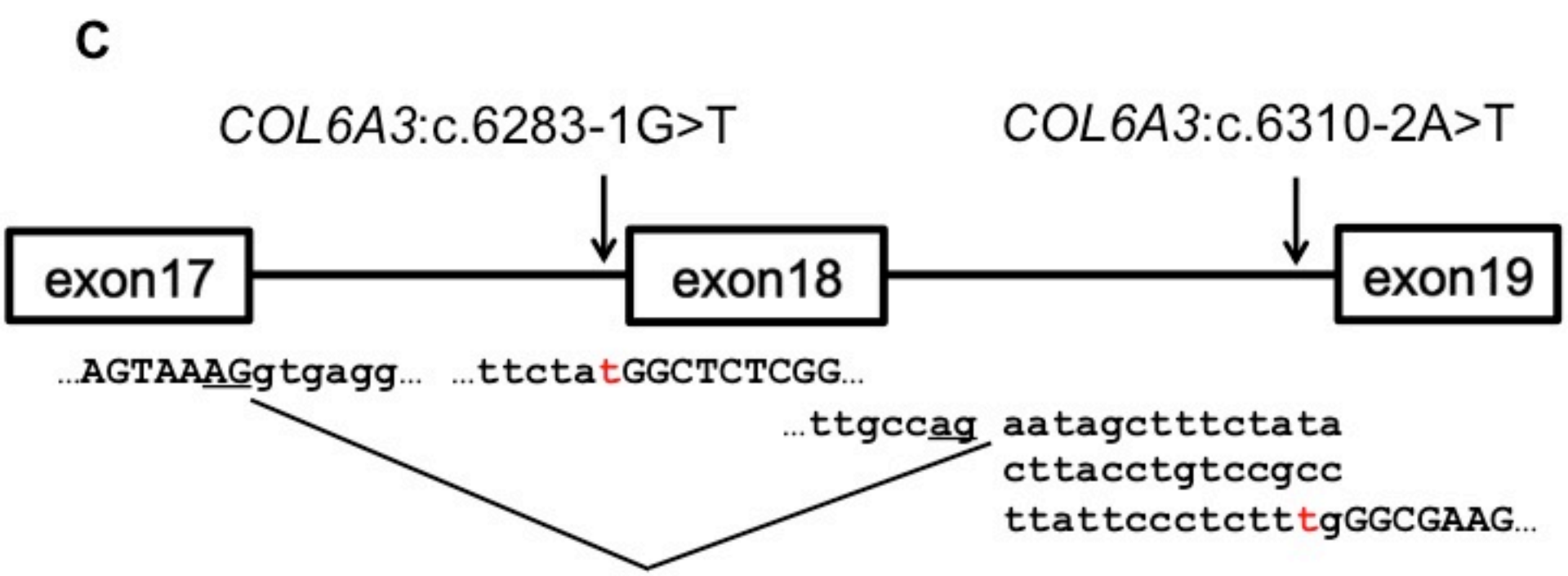


Supplemental Figure 7
Patient with splice site mutation in *COL6A2* (#6). (A-B) Muscle cDNA analysis using primers in exon 6 and 14 showed that normal transcript and aberrant spliced transcript, which skips exon 10, are expressed. C: control. Pt: patient. (C) Exon 10 skipping results in 15-amino acid in-frame deletion. This 15-amino acid deletion disrupts triple-helical region, which is responsible for dominant Ullrich muscular dystrophy.

#7





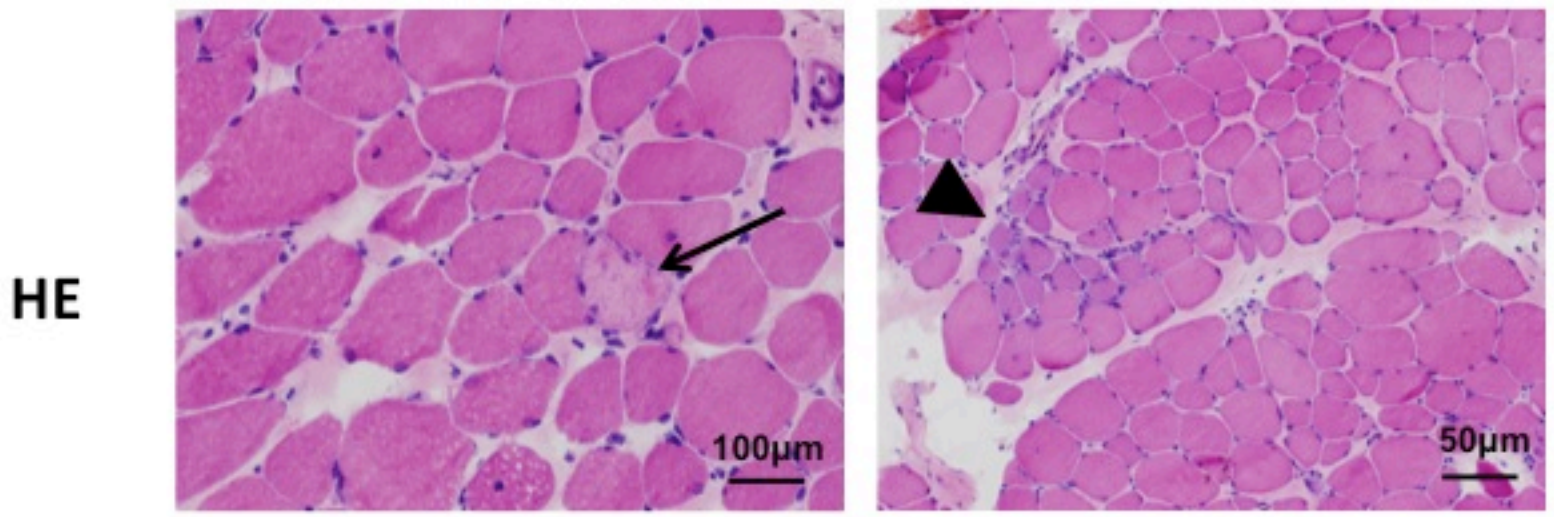


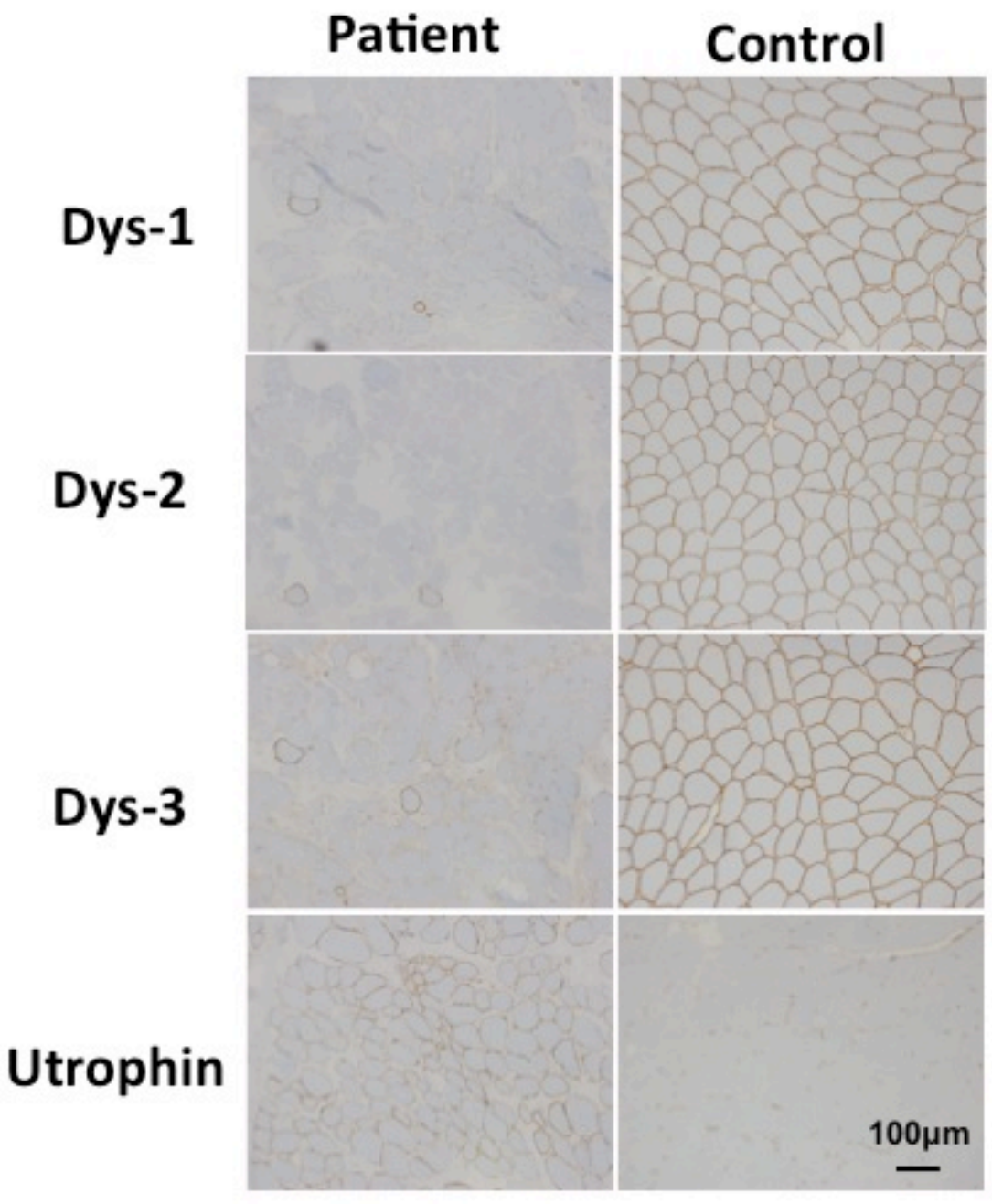
cDNA c.6283_6308delinsAATAGCTTTCTATACTTACCT GTCCGCCTTATTCCCTCTTTG

protein p.(Gly2095_Lys2103delinsAsnSerPheLeuTyrLeu ProValArgLeuIleProSerLeu)

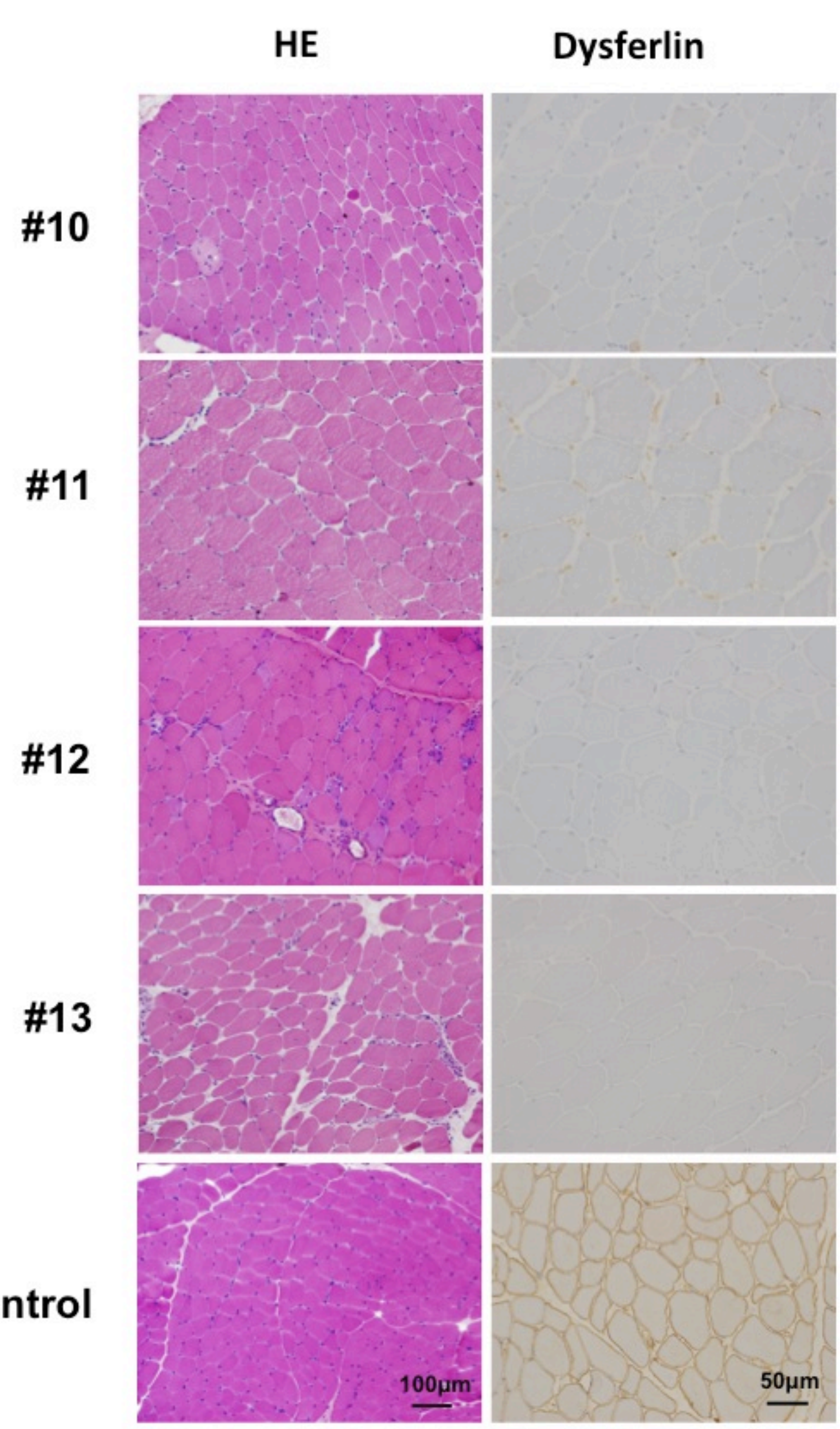
Supplemental Figure 8
Patient with two splice site mutations in *COL6A3* (#7). (A-B) Muscle cDNA amplification using primers in exon 16 and 23-24 showed both normal and aberrantly spliced transcript, which skips exon 18 and the inserted part of intron18-19. C: control. Pt: patient. (C) This transcript is predicted to result in deletion of 9 amino acids and insertion of 14 amino acids. This disrupts the triple-helical region, which is responsible for dominant Ullrich muscular dystrophy.

#9





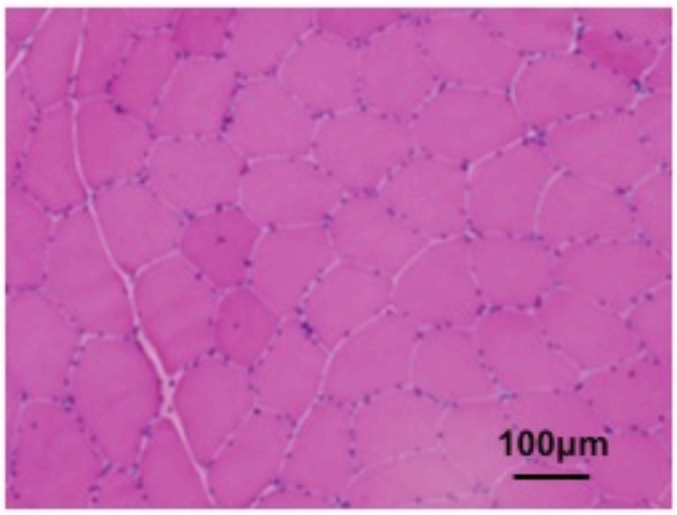
Supplemental Figure 9
HE staining showed dystrophic changes with variation in fiber size, necrotic (arrow) and regenerating fibers (arrowhead), endomysial fibrosis and increased internalized nuclei in a patient with *DMD* mutation (#9). Regenerating fibers are clustered, which is usually seen in dystrophinopathy. Dystrophin did not stain with antibodies against N-terminal (dys-2), rod-domain (dys-1) and C-terminal (dys-3) of dystrophin. Utrophin, which is overexpressed in compensation when dystrophin is absent, was stained in the patient's muscle.



Supplemental Figure 10
HE staining of samples from patients and control with *DYSF* mutations (#10-13) showed dystrophic changes. The expression of dysferlin was not detected by immunohistochemical staining.

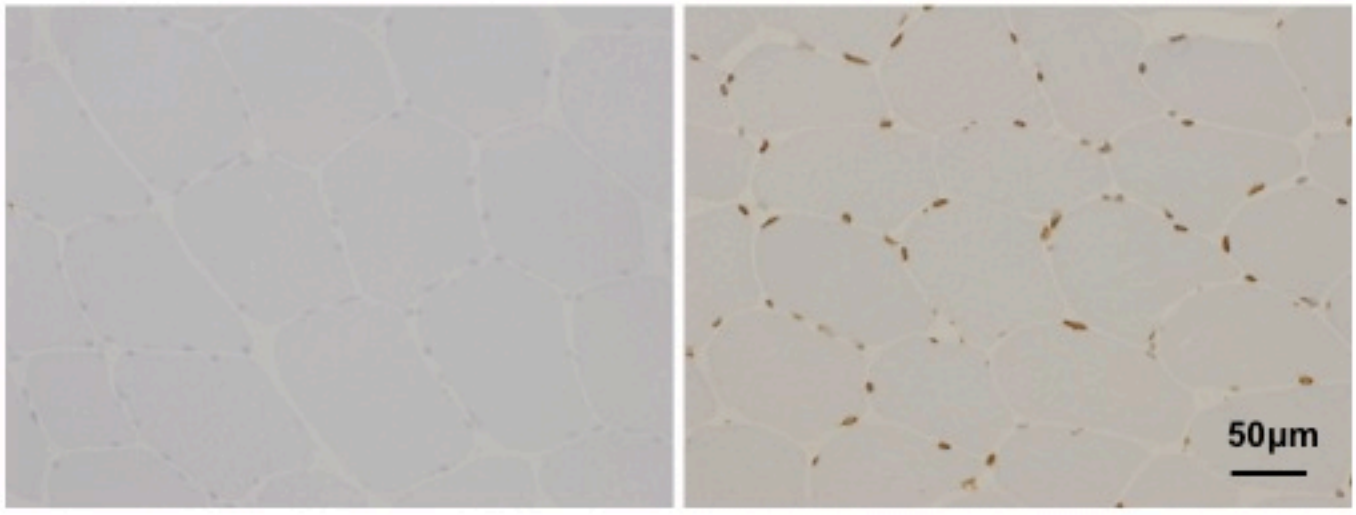
#14

HE

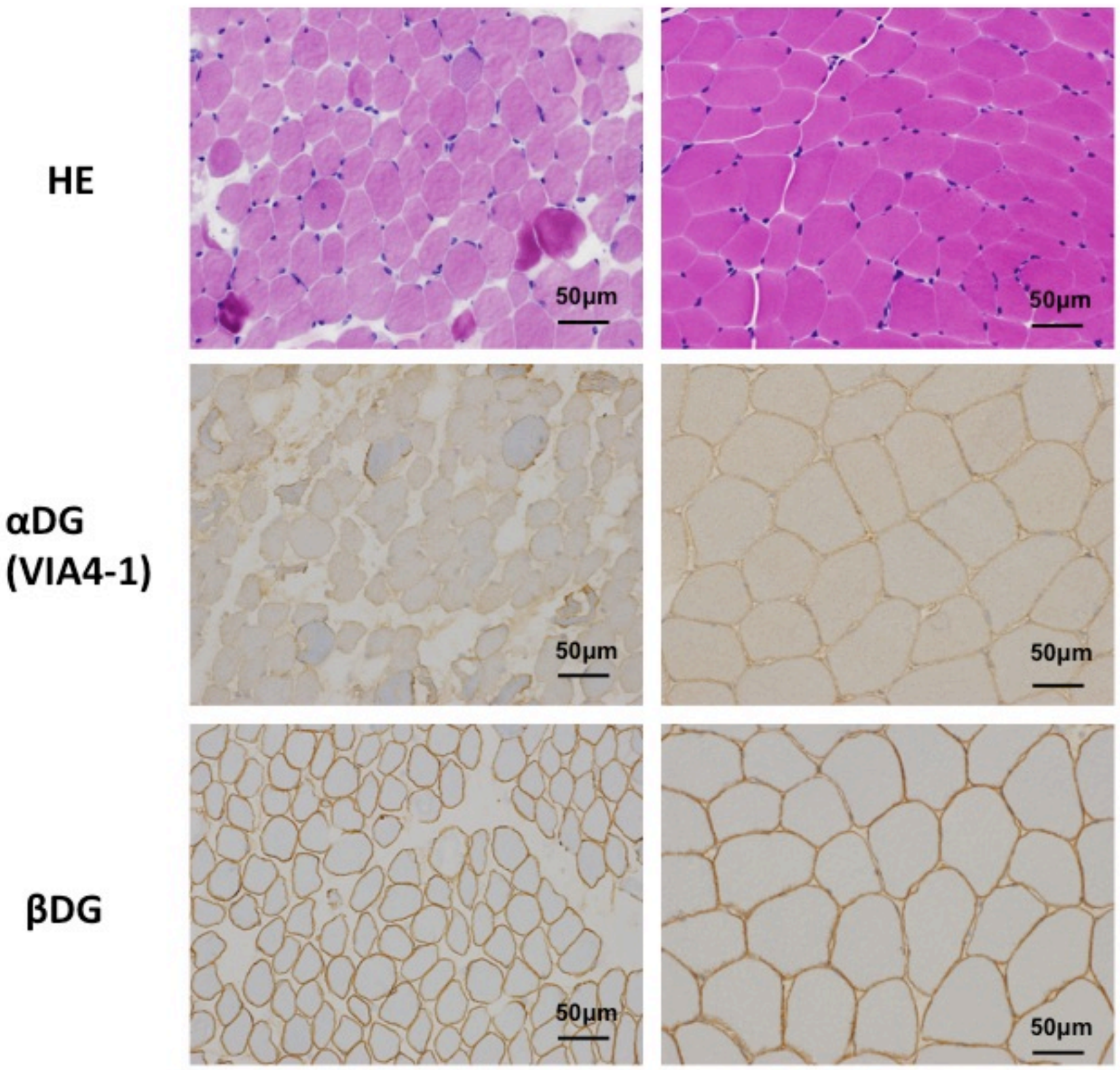


Control

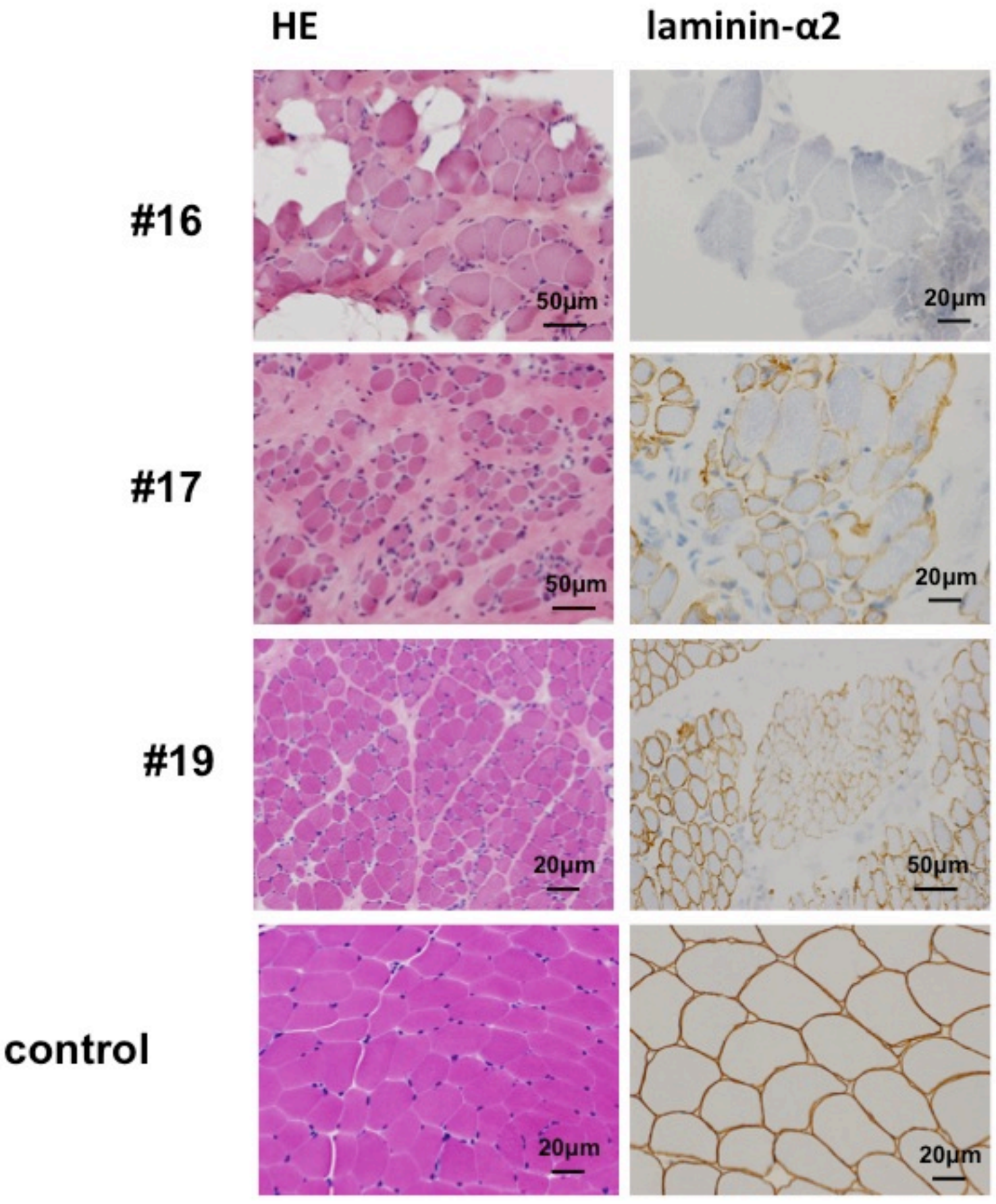
Emerin



Supplemental Figure 11
HE staining in patient's muscle with *EMD* mutations (#14) showed nonspecific changes with mild variation in fiber size and occasional fibers with internalized nuclei. Emerin staining was absent, confirming emerinopathy.



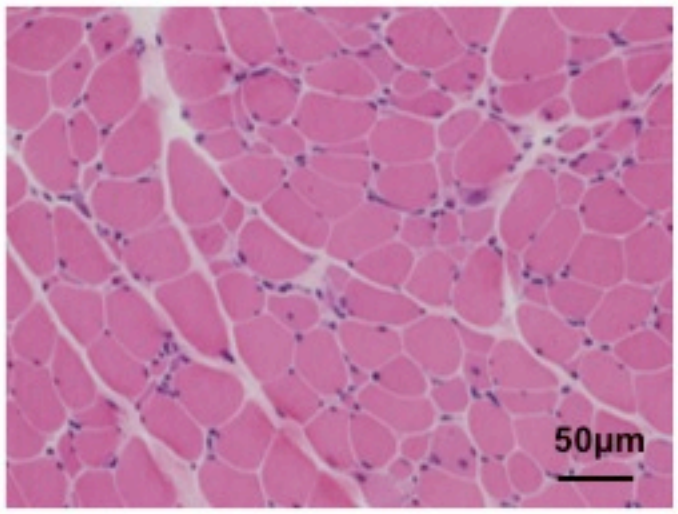
Supplemental Figure 12
HE staining in patient's muscle with a *FKTN* mutation (#15) showed dystrophic changes, including an increased number of internalized nuclei and endomysial fibrosis. Immunohistochemical staining of glycosylated alpha dystroglycan (VIA4-1) showed decreased staining in the sarcolemma, suggesting that the patient has alpha dystroglycanopathy. Western blotting using the same antibody also showed decreased glycosylation (data not shown).



Supplemental Figure 13
HE staining in patients' muscles with *LAMA2* mutations revealed the advanced stage of degeneration with marked endomysial fibrosis and adipose tissue infiltration (#16 and #17) or moderate variation in fiber size and endomysial fibrosis (#19). Necrotic and regenerating fibers were not prominent. Laminin alpha2 staining showed complete (#16) and partial (#17 and #19) deficiency.

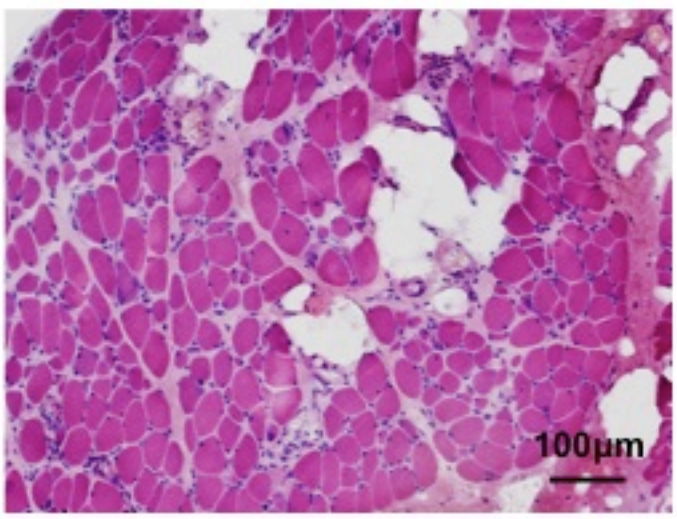
#20

HE



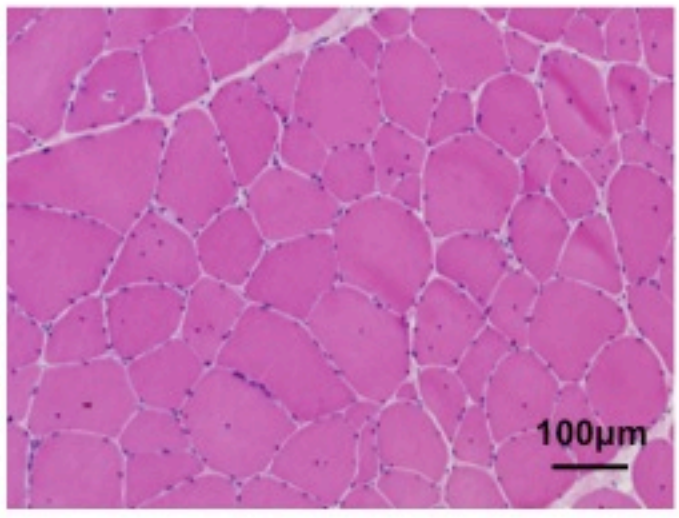
#21

HE

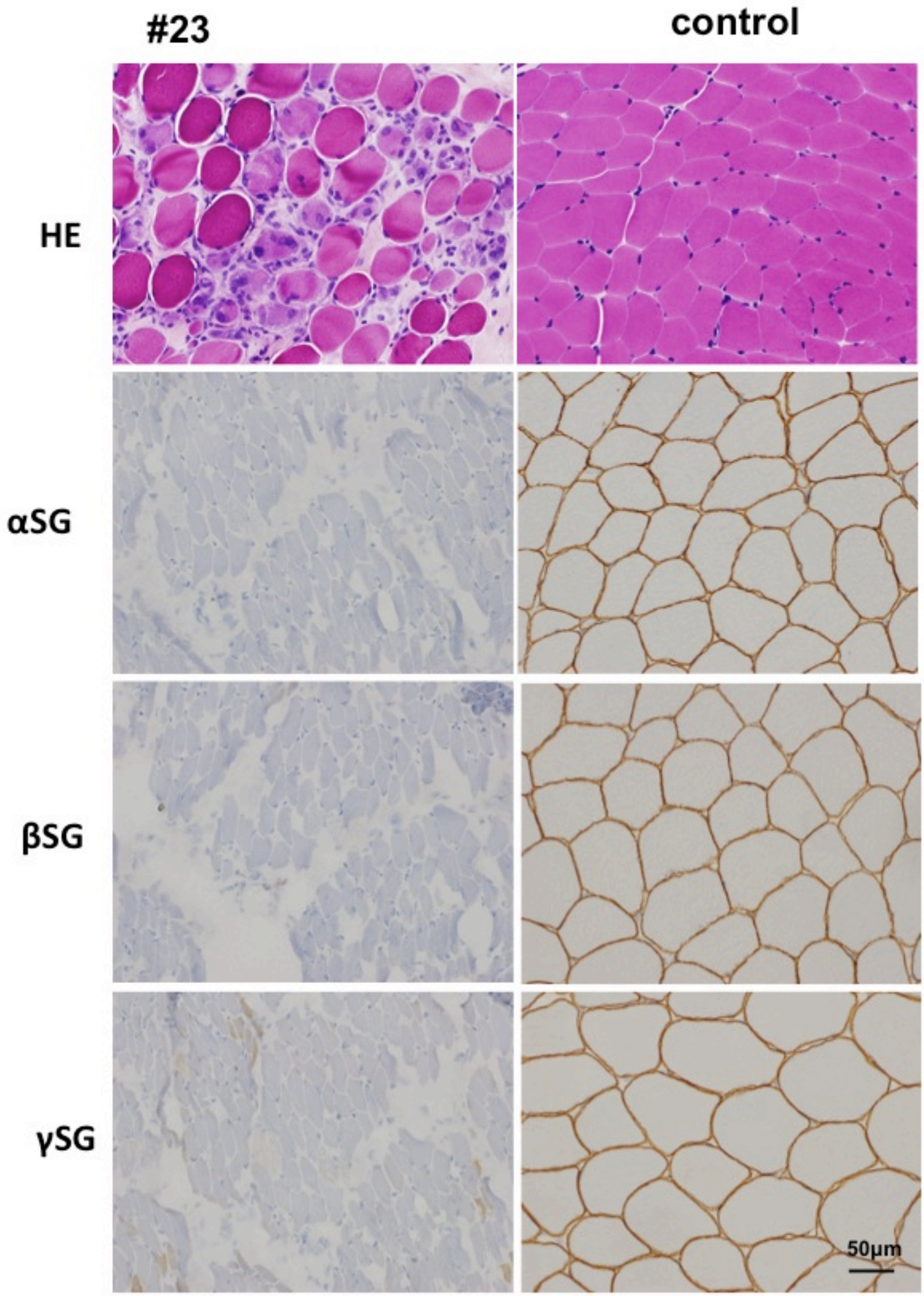


#22

HE

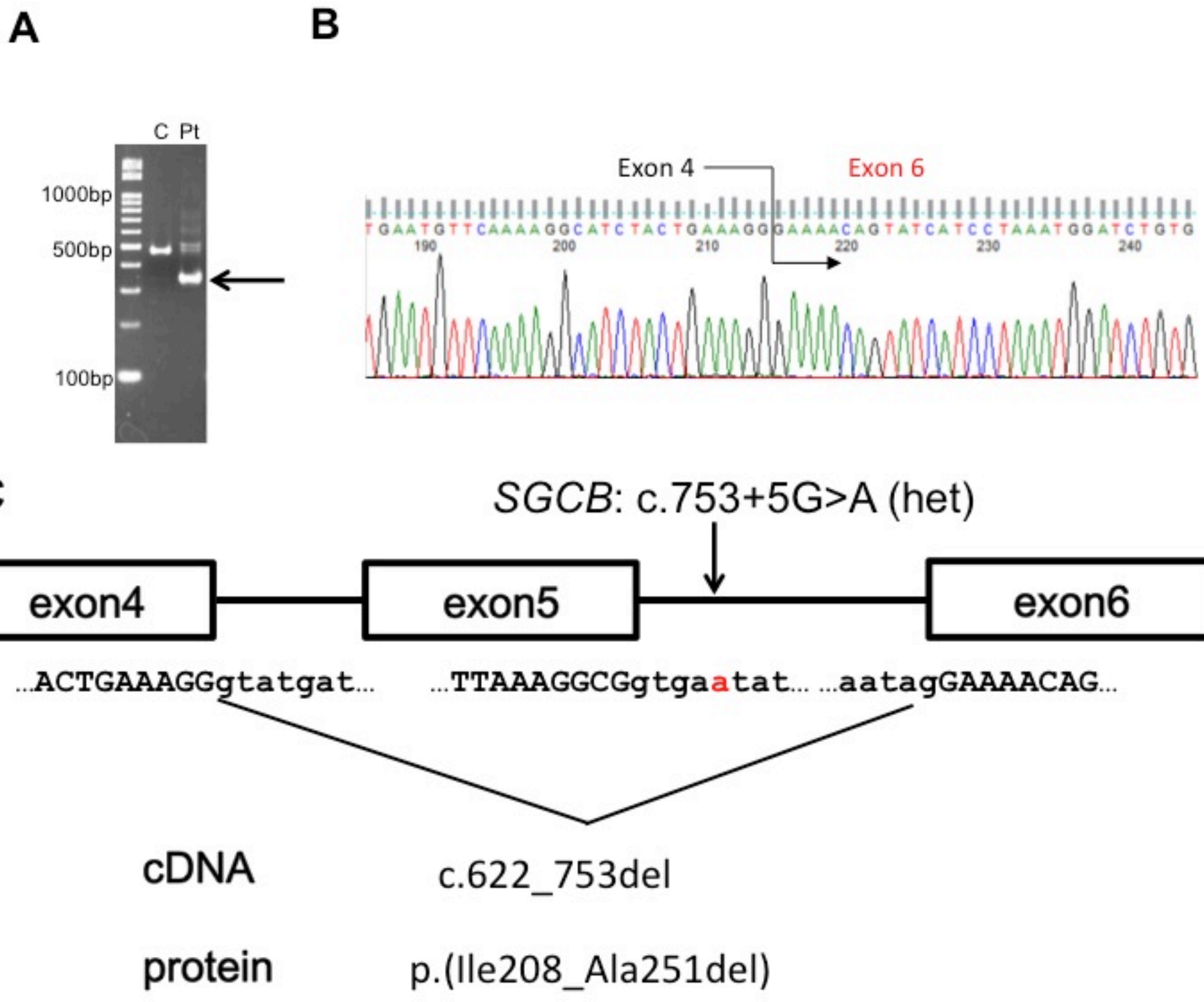


Supplemental Figure 14
HE staining of patients' muscle with *LMNA* mutations, all patients showed an increased number of internalized nuclei. Patients #20, #21, and #22 exhibited variations in fiber size and slightly increased endomysial fibrosis. Patient #21 displayed advanced dystrophic changes including fibrosis and adipose tissue replacement. Necrotic and regenerating fibers were not prominent in all patients.

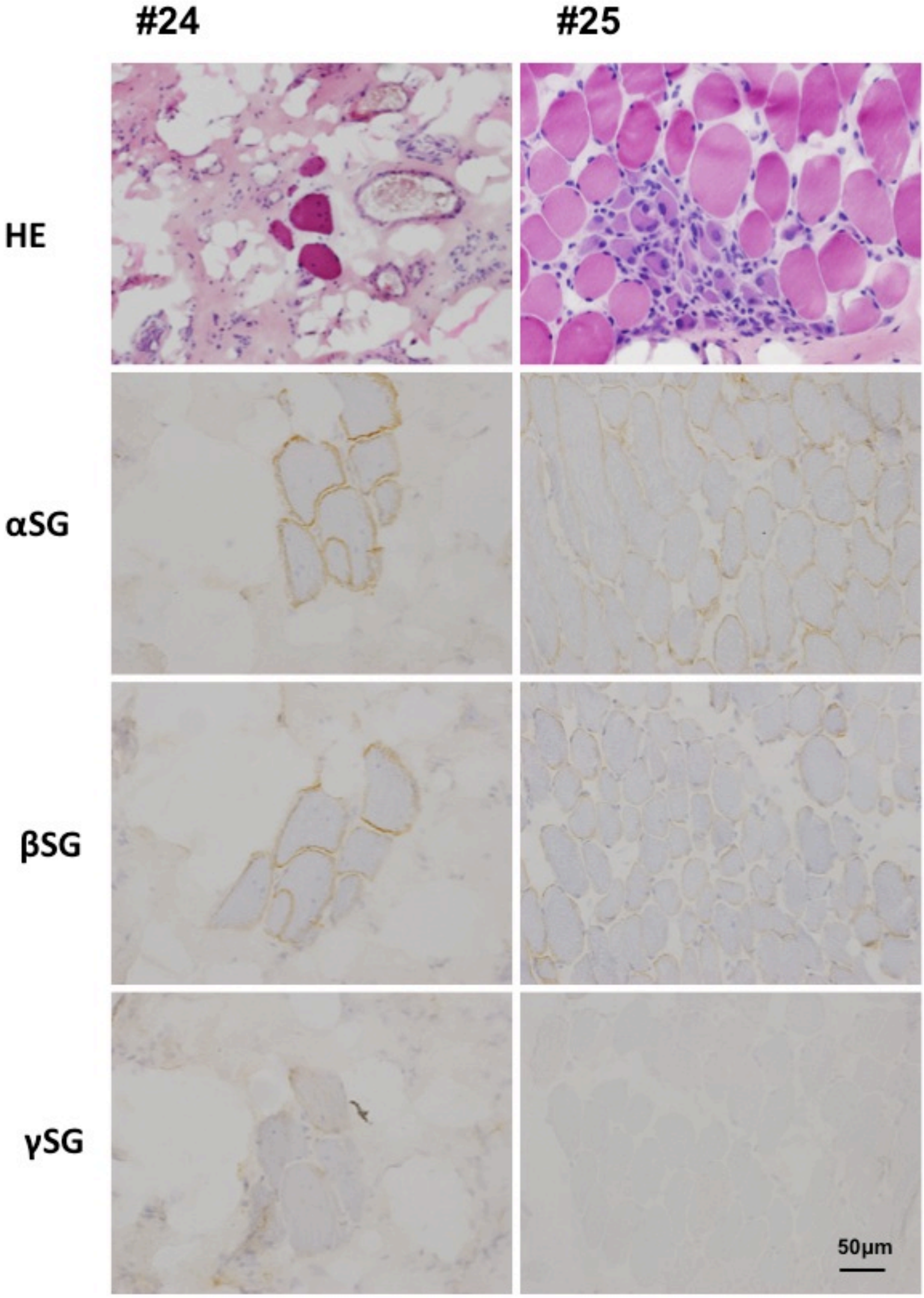


Supplemental Figure 15
Patient #23 had splice-site and stop codon mutations in *SGCB*. The patient was deficient in sarcoglycan alpha, beta and gamma.

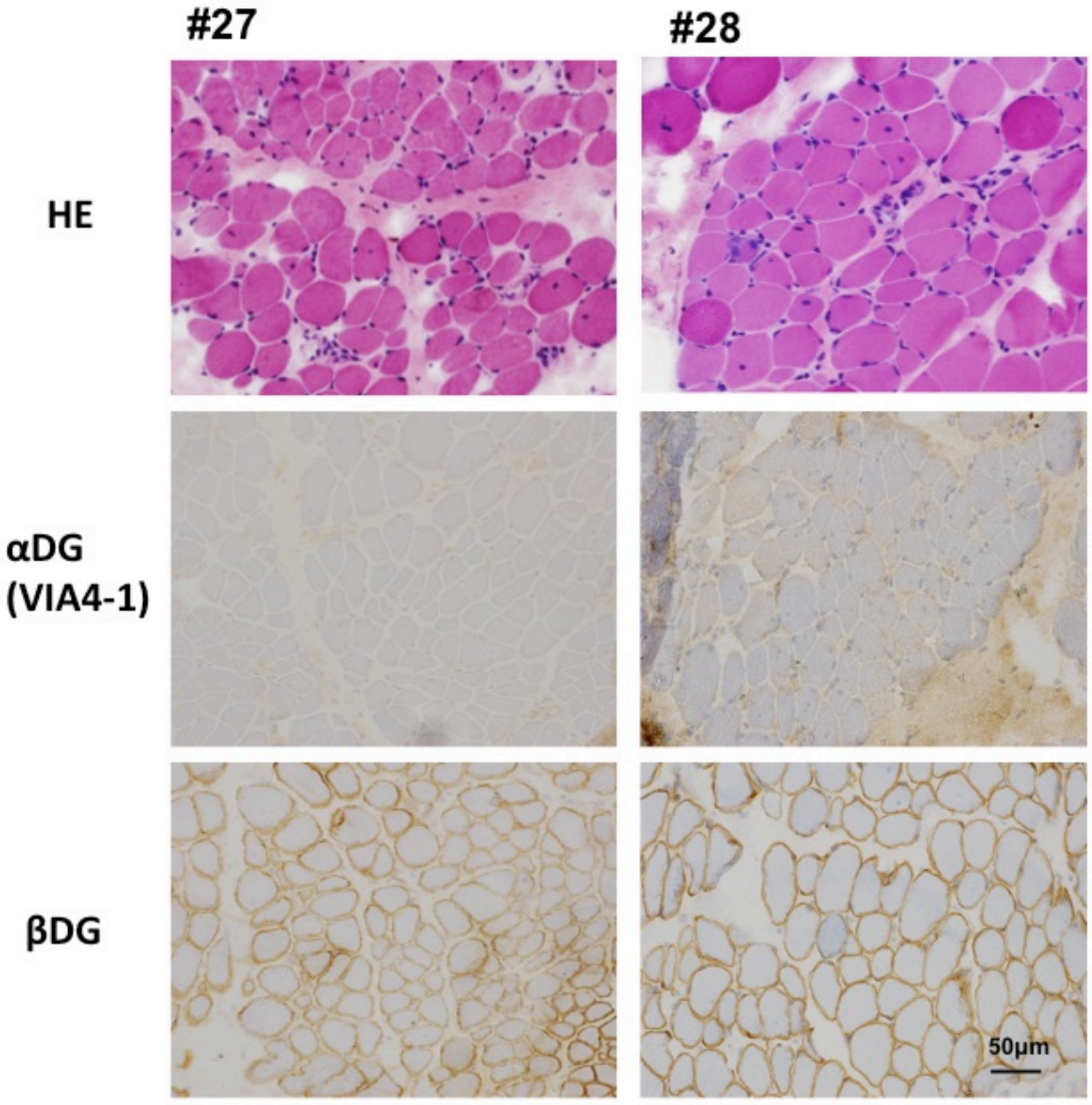
#23



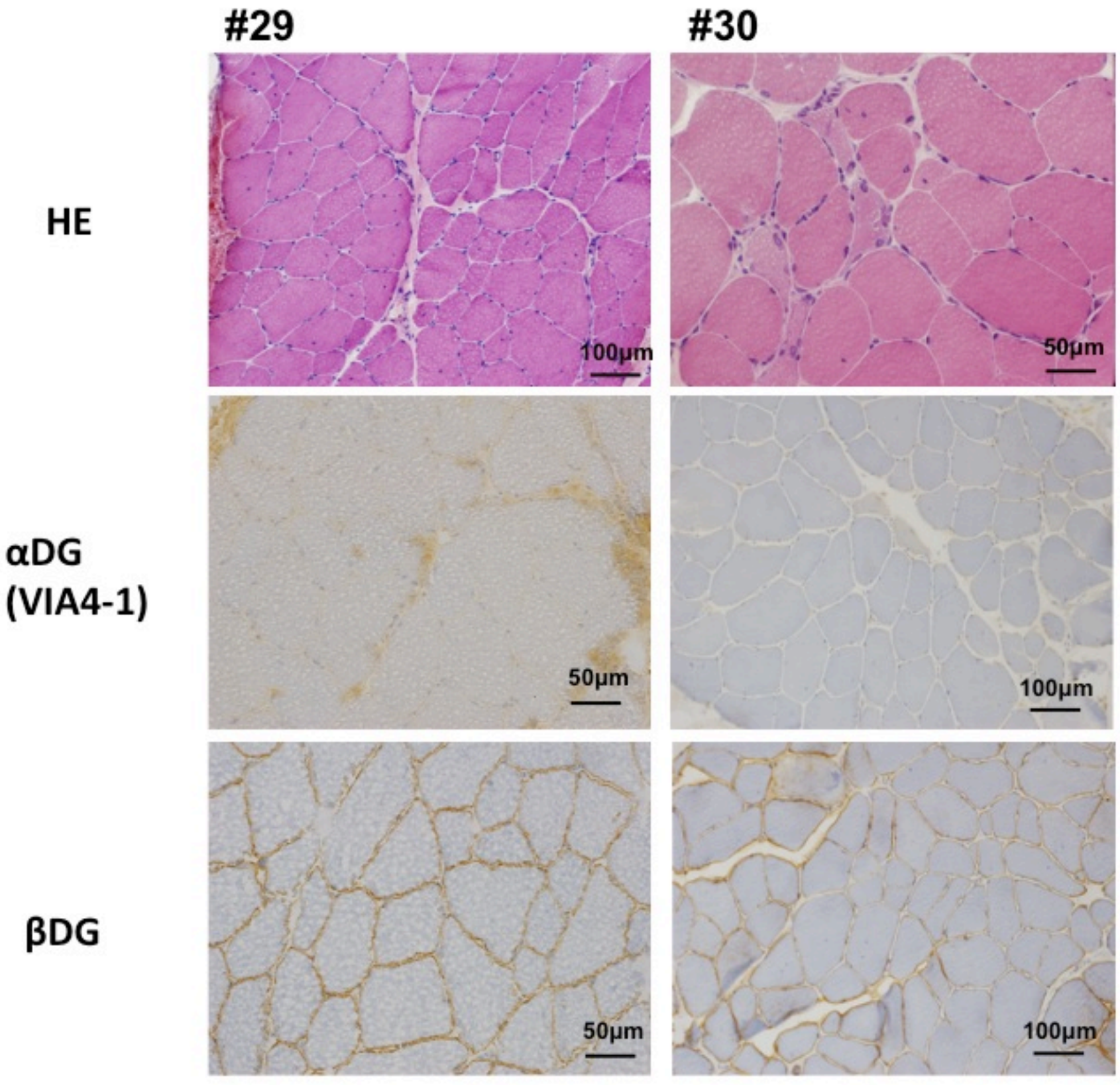
Supplemental Figure 16
Patient #23 had a splice-site (c.753+5G>A) and stop codon mutations (R109*) in *SGCB*. (A) Muscle cDNA was amplified using primers at exon 3 and 6. Short and normal sized PCR products were observed. C: control. Pt: patient. (B) Sequencing analysis of each band showed exon 5 skipping and a normal transcript. (C) It is likely that the splice-site mutation causes exon 5 skipping.



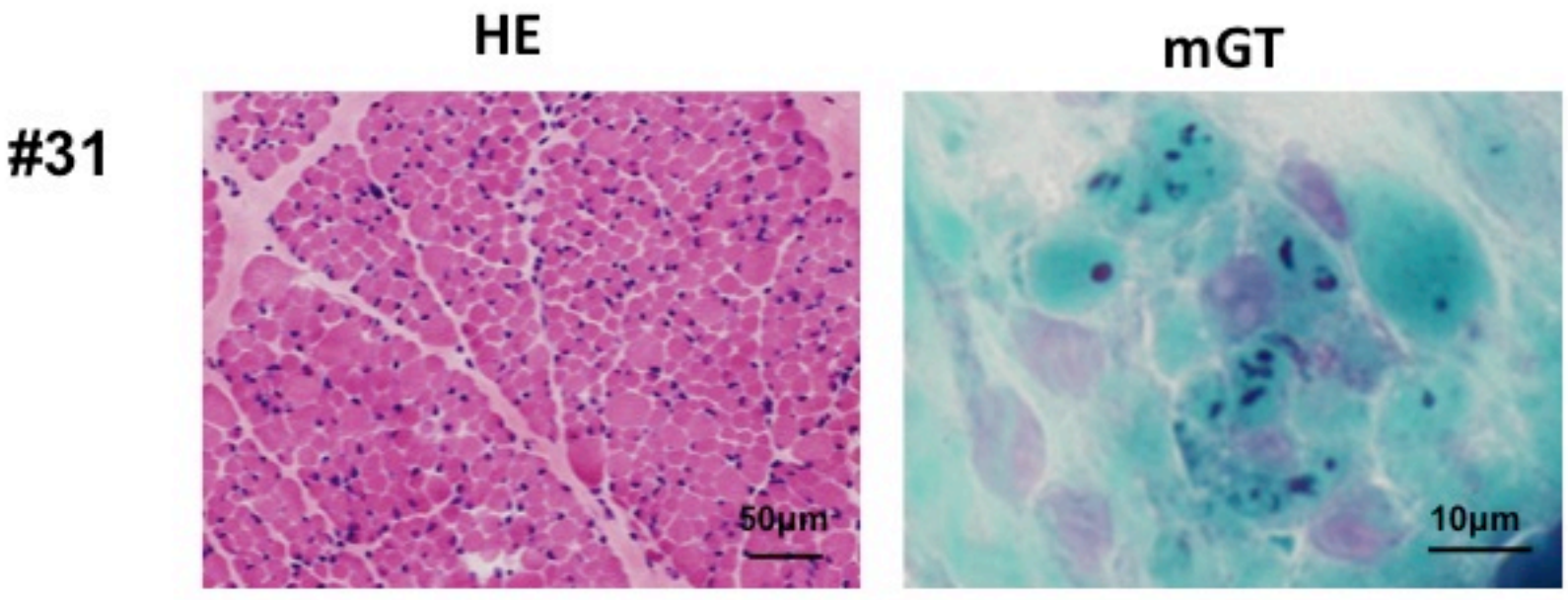
Supplemental Figure 17
Patients with *SGCG* mutations. HE staining showed advanced muscle damage in patient #24 and dystrophic changes with necrotic and regenerating fibers in #25. Alpha and beta sarcoglycans were decreased, while gamma sarcoglycan was not stained in the patients' muscles.



Supplemental Figure 18
Patients with *POMGNT2* mutations (#27, 28). HE staining showed variations in fiber size and increased internalized nuclei and endomysial fibrosis. Immunohistochemical staining of glycosylated alpha-dystroglycan using VIA4-1 antibody showed decreased glycosylation, while staining for beta-dystroglycan protein is preserved. Western blotting analysis using the same antibody also showed the alpha-dystroglycan glycosylation defect (data not shown).

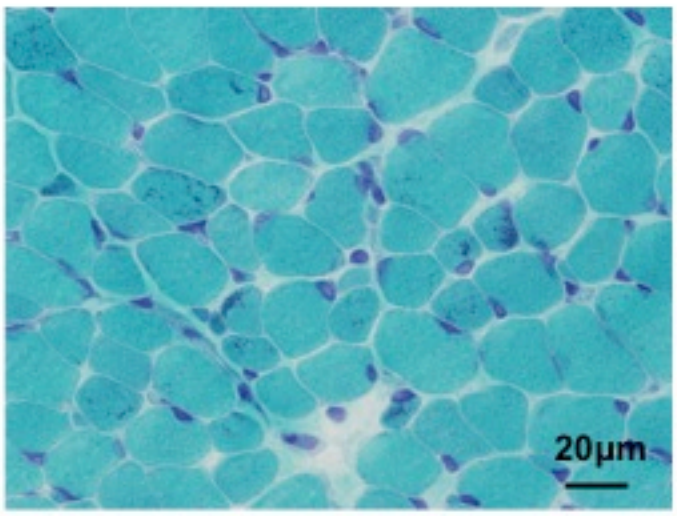


Supplemental Figure 19
Patients with *POMT2* mutations (#29 and #30). HE staining showed variations in fiber size and increased internalized nuclei. Immunohistochemical staining of glycosylated alpha-dystroglycan using VIA4-1 antibody showed decreased glycosylation while staining for beta-dystroglycan protein is preserved. Western blotting analysis using the same antibody also showed alpha-dystroglycan glycosylation defect (data not shown).

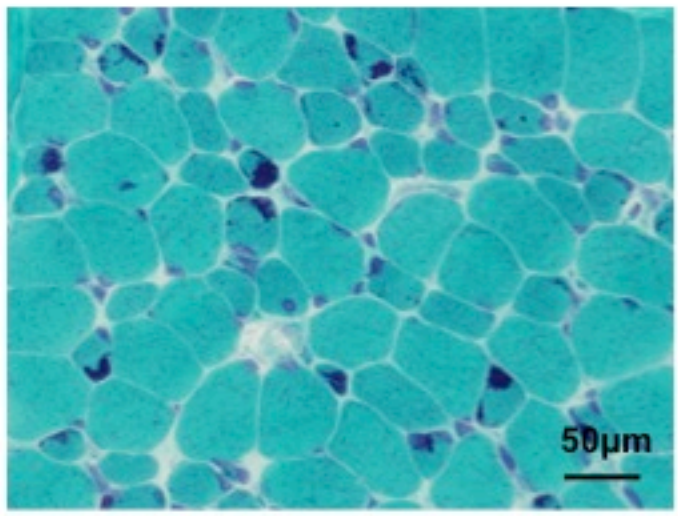


Supplemental Figure 20
Three-month old patient with nemaline myopathy with *ACTA1* mutation, p.N94K (#31). HE staining showed extremely small muscle fibers. In modified Gomori-trichrome staining, nemaline and cytoplasmic bodies were seen. Intranuclear rod was not observed.

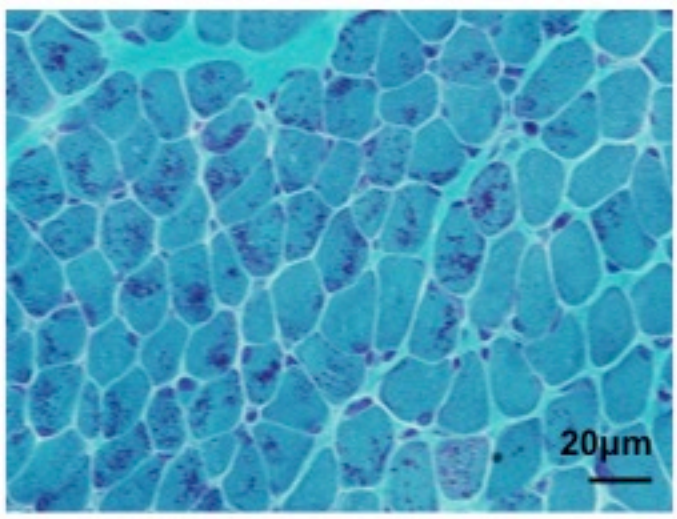
#33



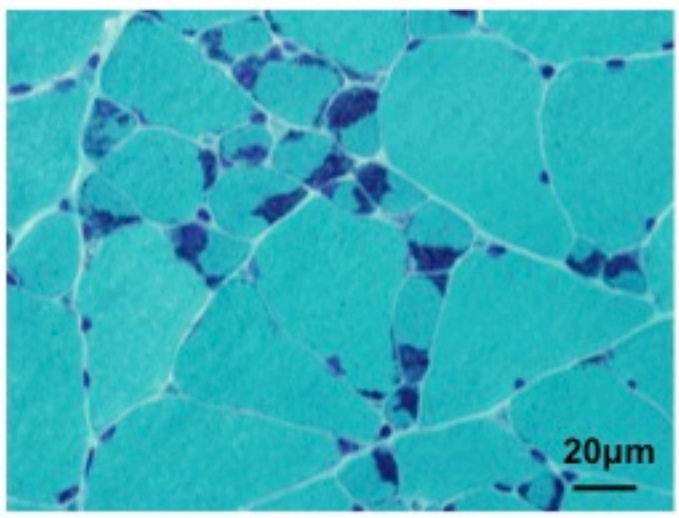
#35



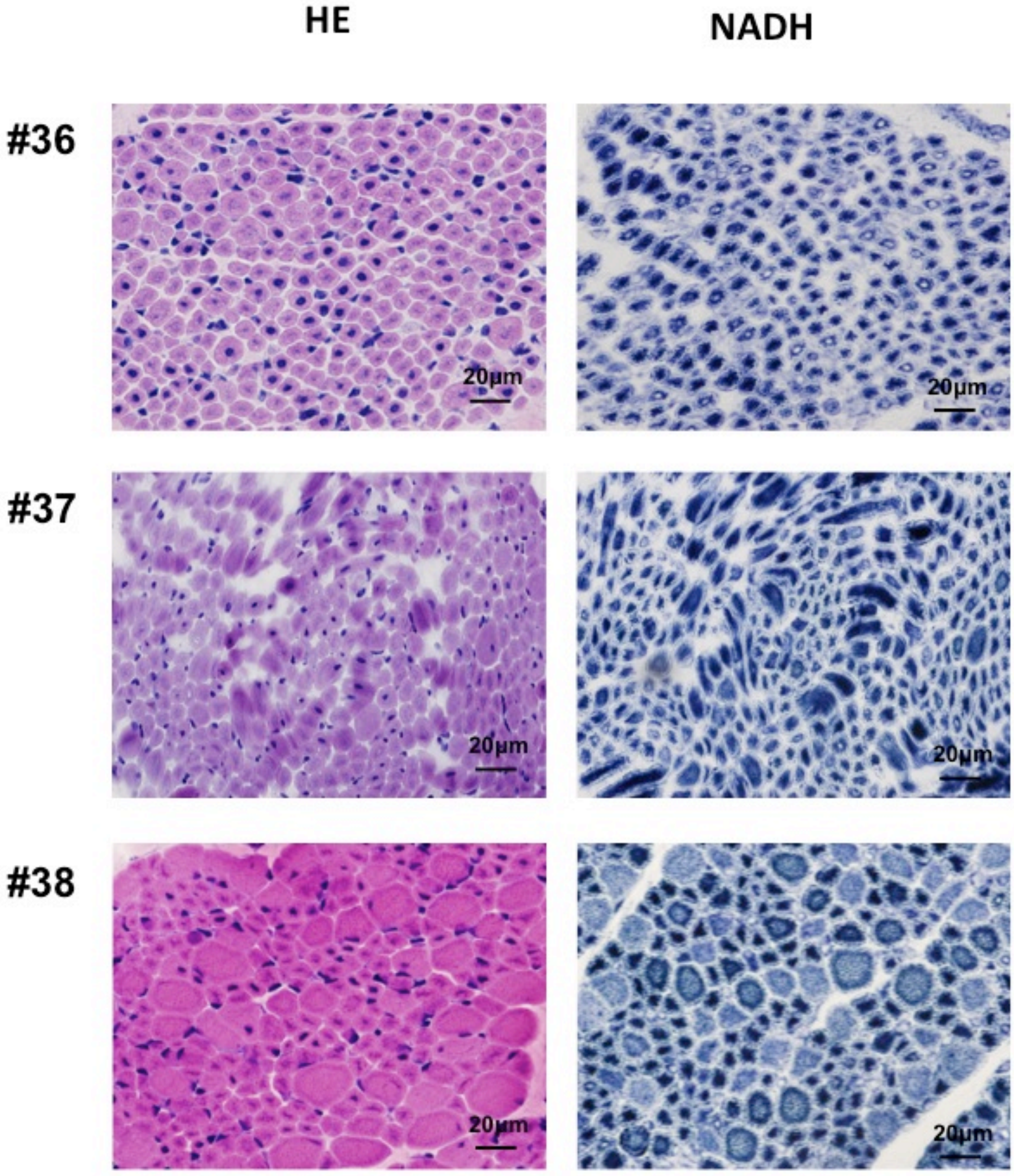
#34



#46

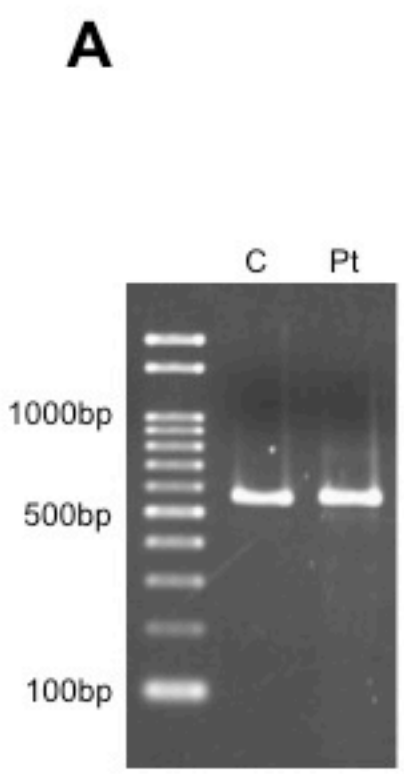


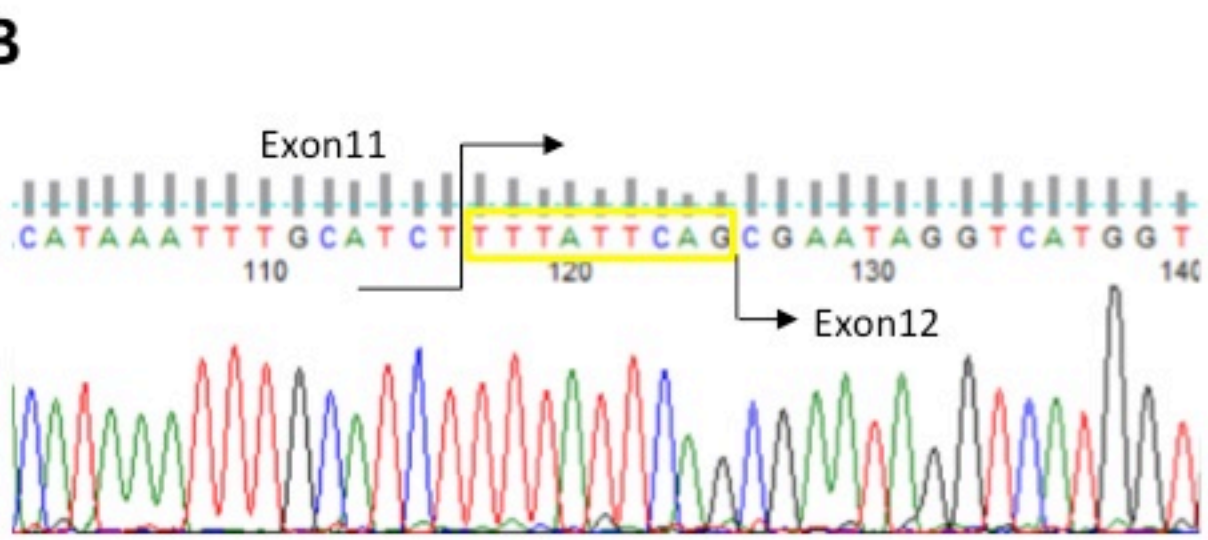
Supplemental Figure 21
Modified Gomori-trichrome staining of nemaline myopathy with mutations in *KLHL40* (#33 and #34), *NEB* (#35) and *TPM2* (#46). All patients showed nemaline rods in cytoplasm.

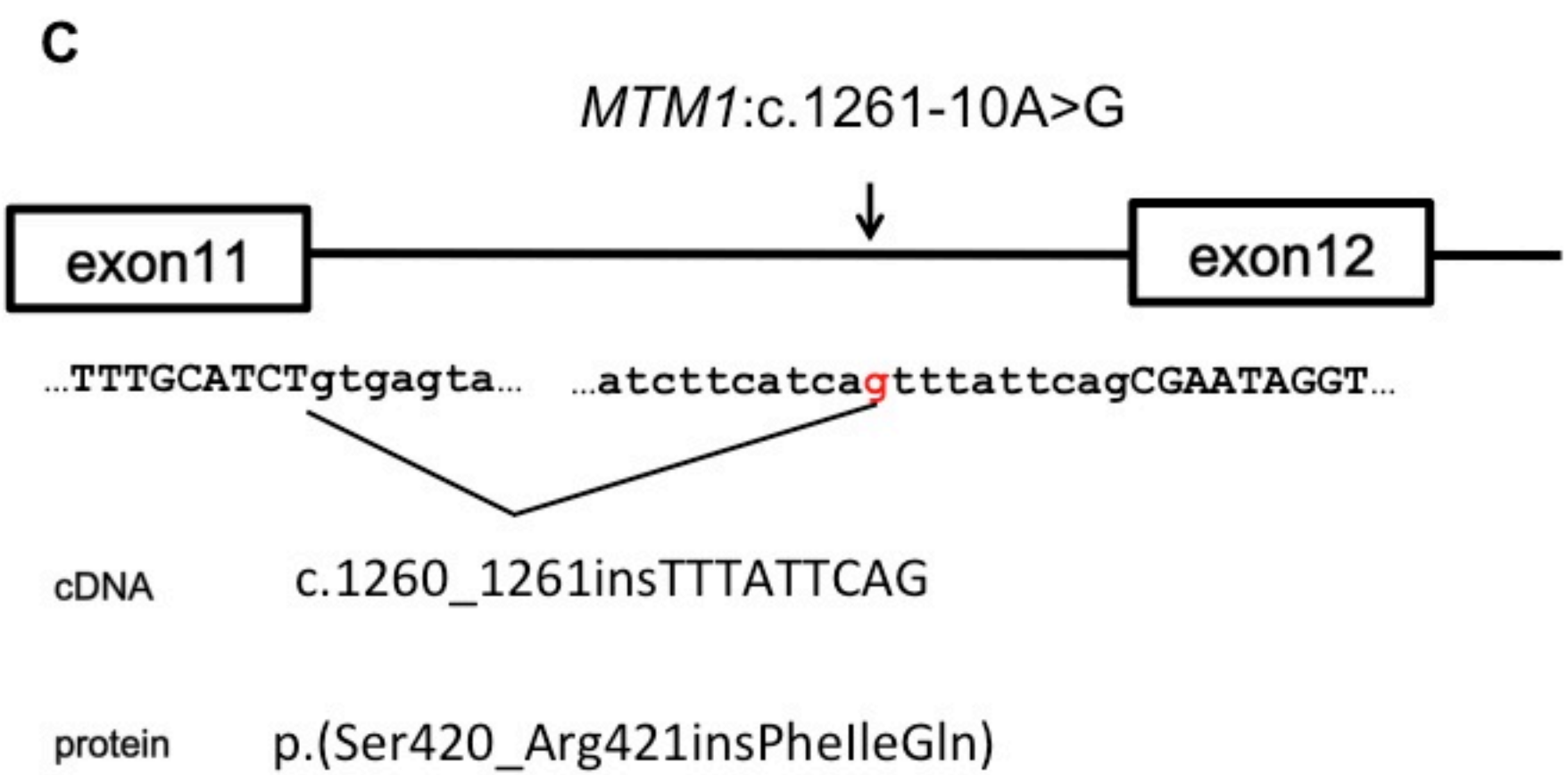


Supplemental Figure 22
Patients with *MTM1* mutations (#36-38). HE staining showed small muscle fibers. NADH staining showed myotubular appearance.

#36

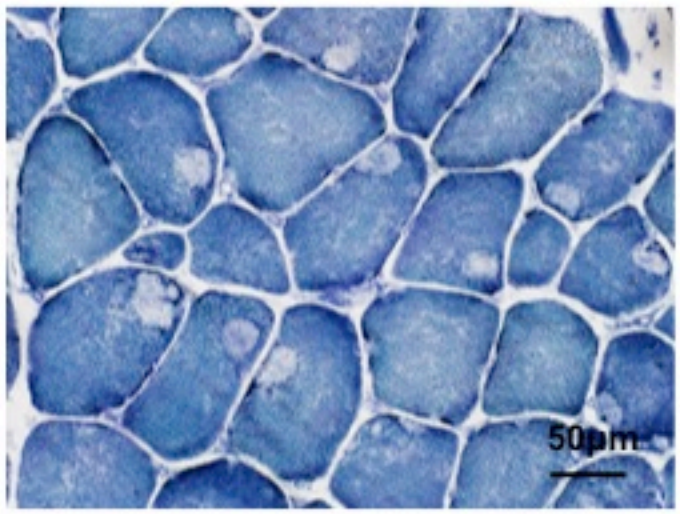




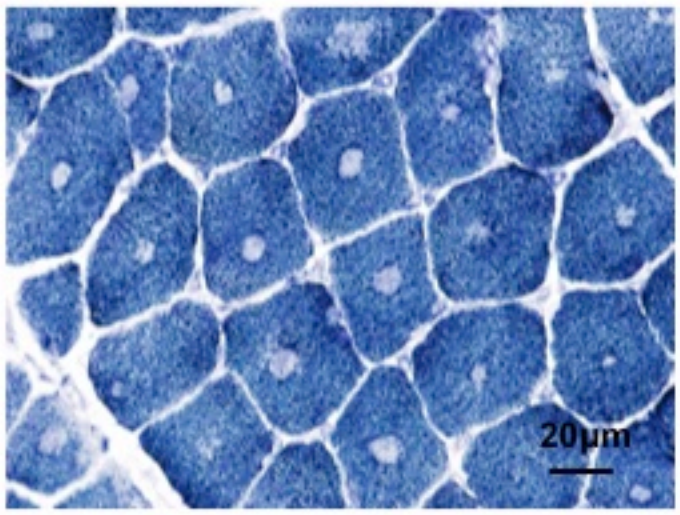


Supplemental Figure 23
Muscle derived cDNA of patient #36 amplified using primers at exon 11 and 14-15 revealed insertion of 9 nucleotides due to creation of new splicing acceptor site. This altered splicing is predicted to cause 3 amino acids insertion as reported previously in the patient with the same mutation (28). C:control. Pt:patient.

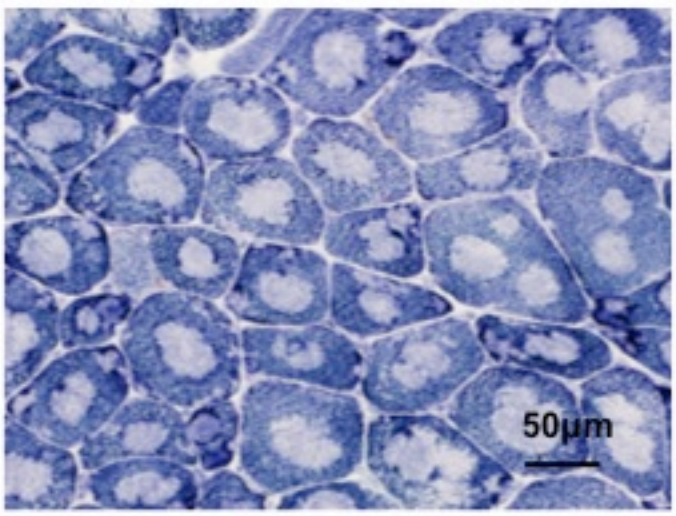
#39



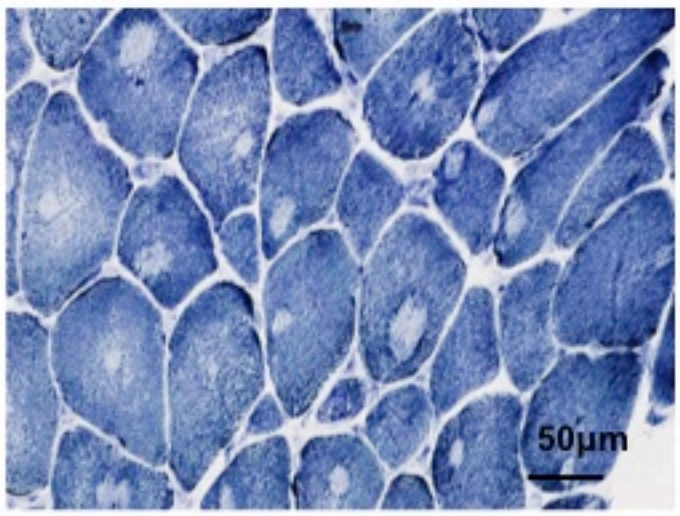
#42



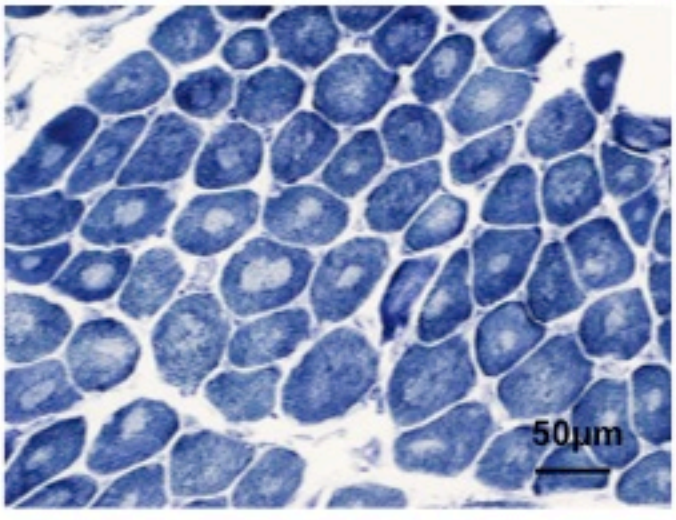
#40



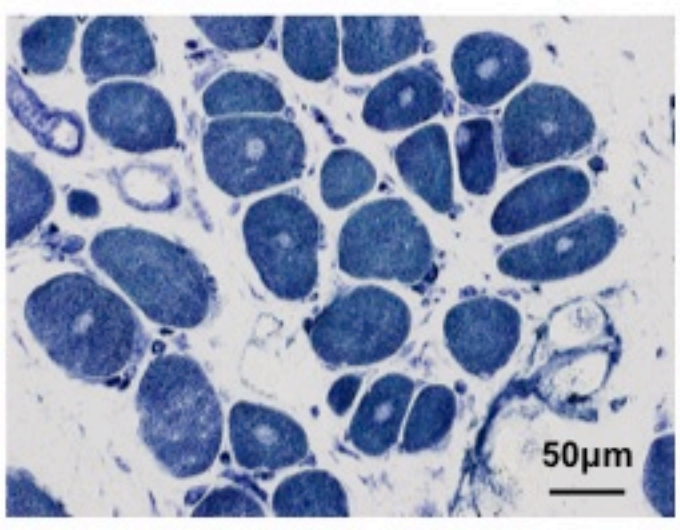
#43



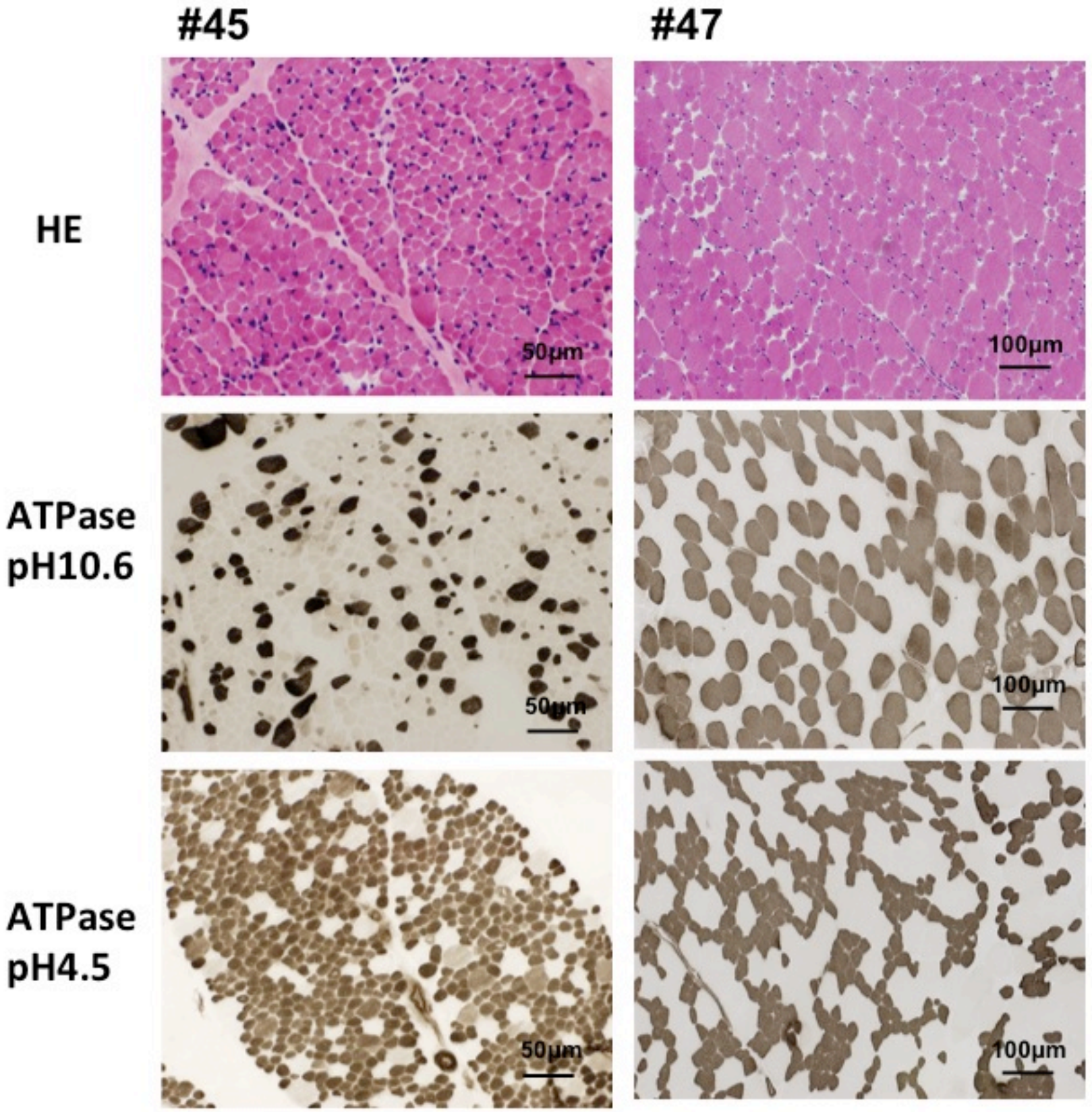
#41



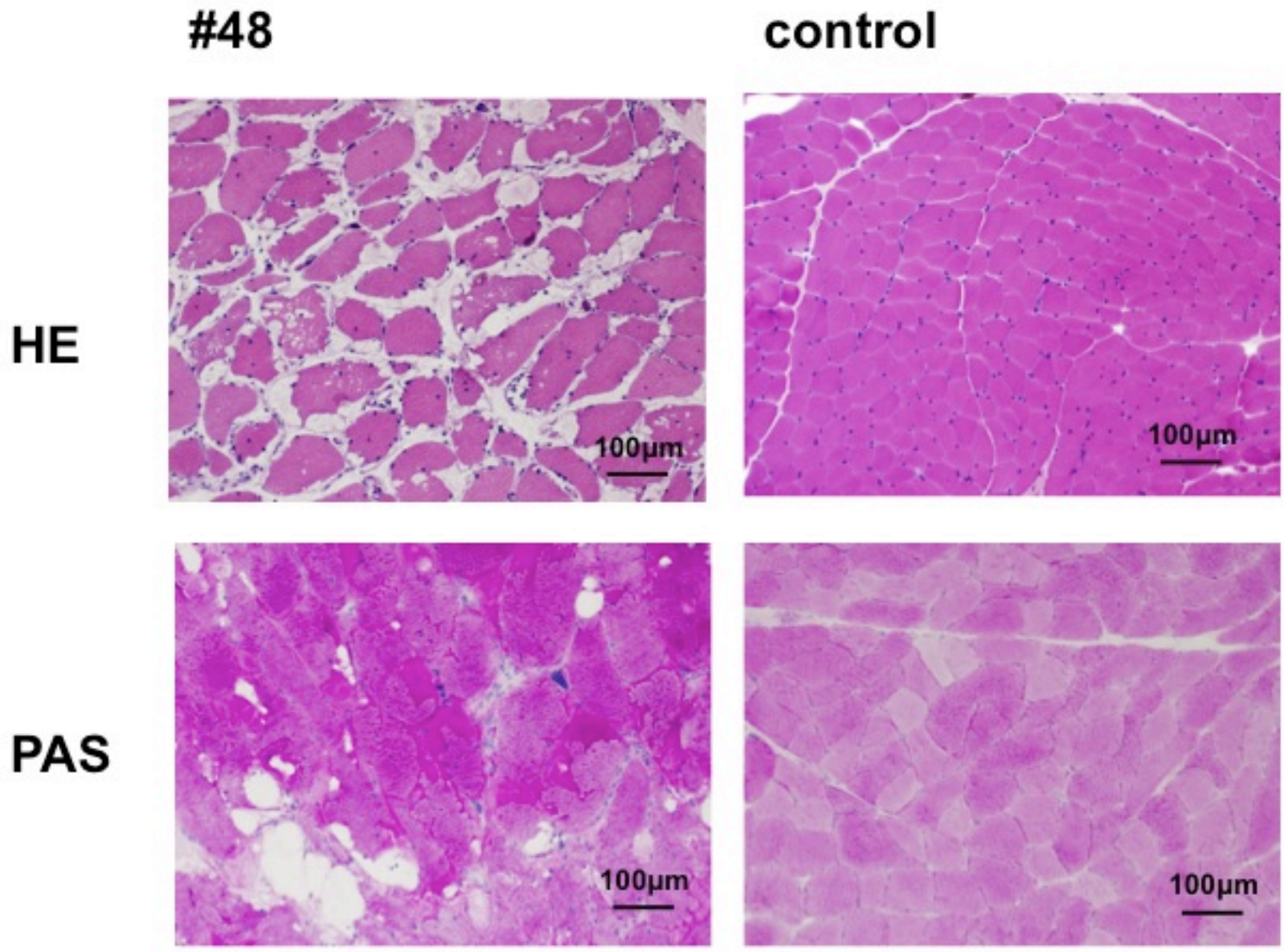
#44



Supplemental Figure 24
Patients #39-44 harbored mutations in *RYR1*, which is known to cause central core myopathy. NADH staining of biopsied muscle all showed central core structure.



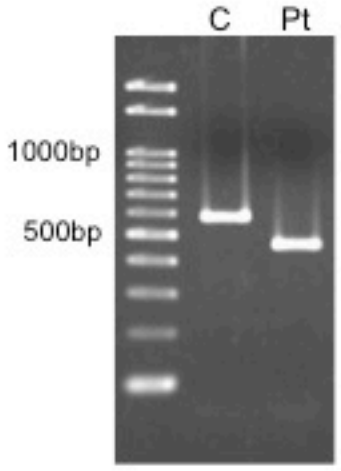
Supplemental Figure 25
Patients with *RYR1* (#45) and *TPM3* (#47) mutations. HE staining showed variation in fiber size and occasional fibers with internalized nuclei. In myosin ATPase staining at pH4.5, which stains type 1 fibers dark, type1 fibers were extremely small. This finding corresponded to the pathology of CFTD.



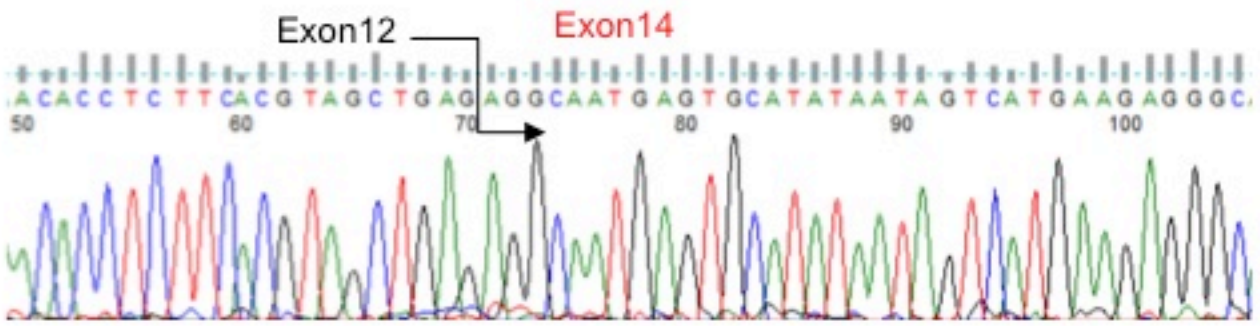
Supplemental Figure 26
HE staining in a patient' muscle with AGL mutation (# 48) showed vacuolated fibers in subsarcolemmal areas. PAS staining showed glycogen accumulation.

#48 *AGL* c.1735+1G>T (hom)

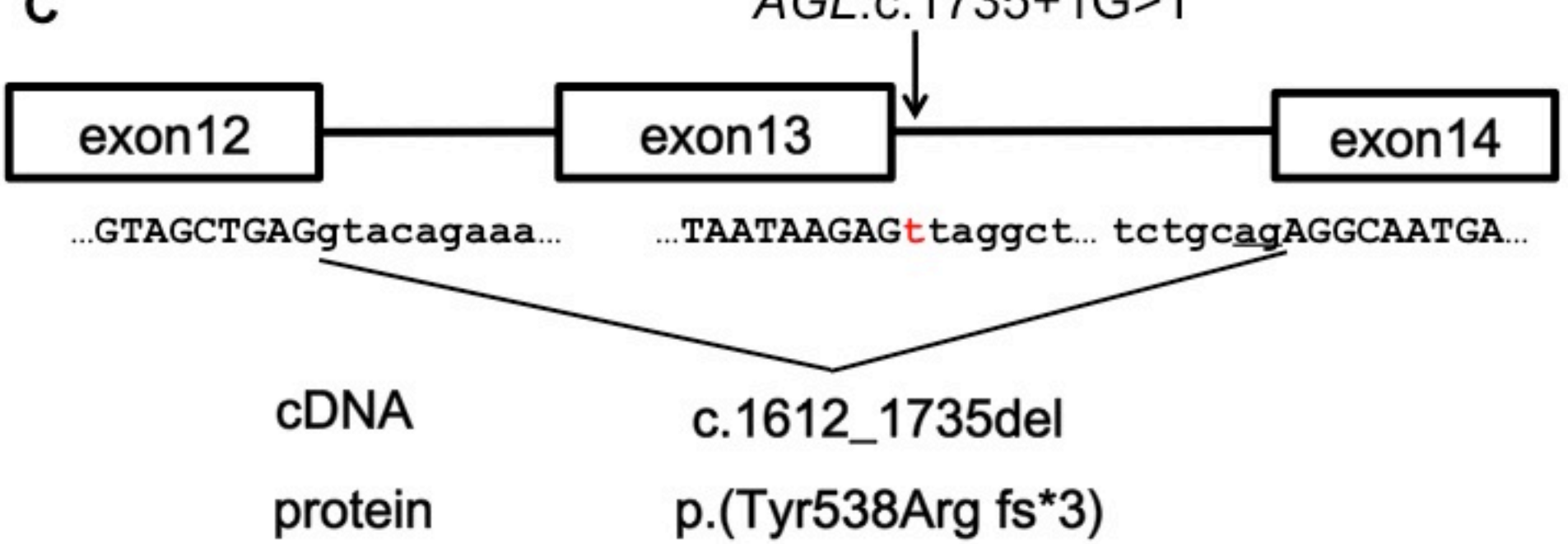
A



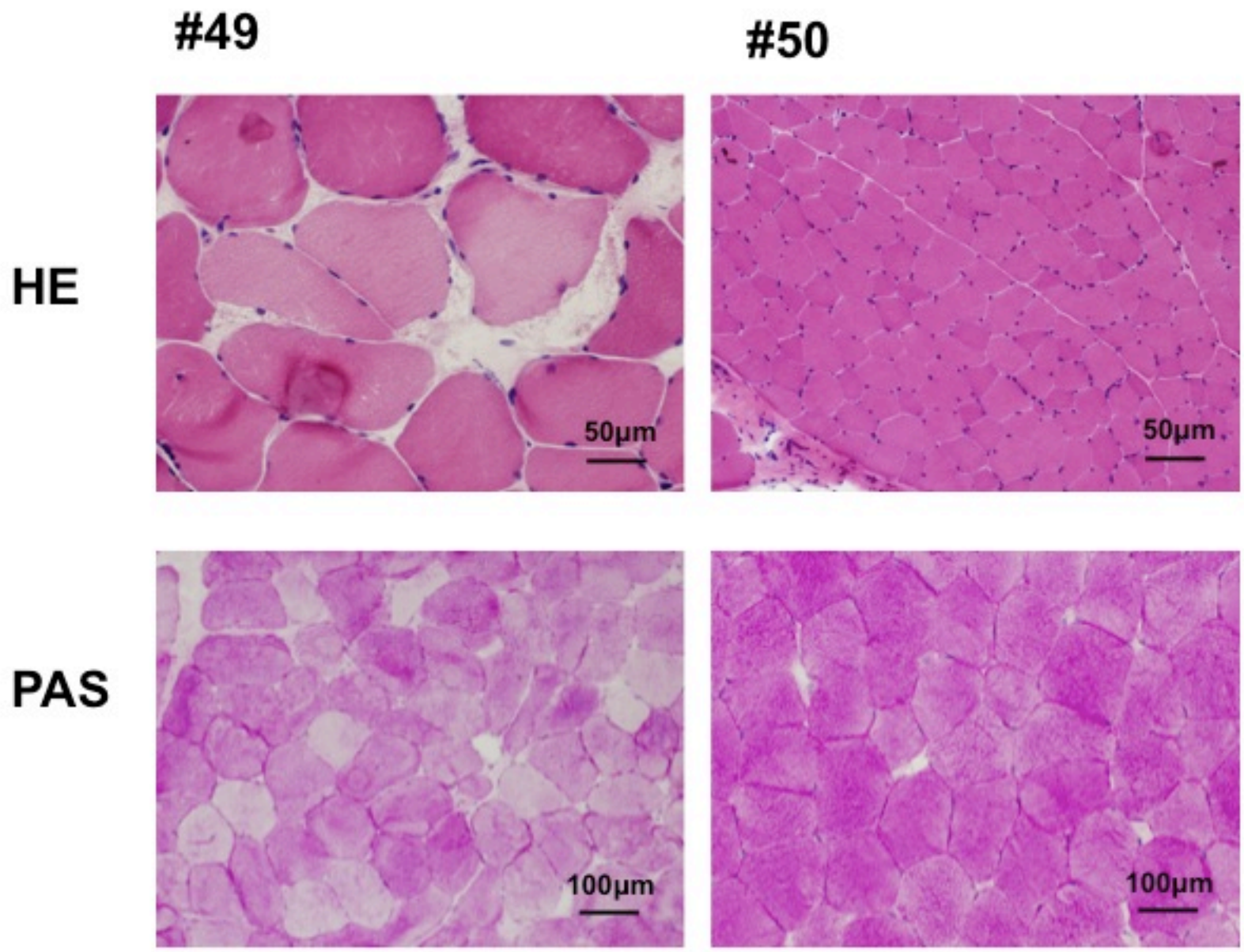
B



C

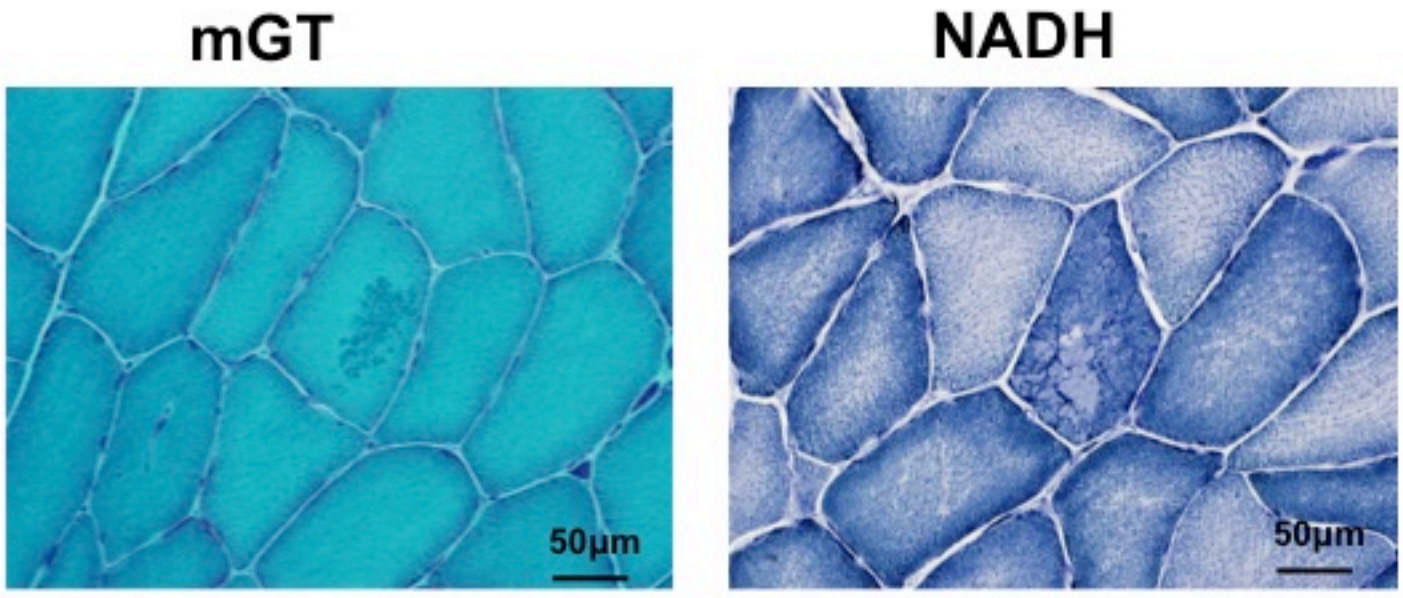


Supplemental Figure 27
(A-B) Muscle derived cDNA of a patient #48 was amplified using primes in exon 10 and 14. Shorter transcript were only expressed in the patient compared to control. Sequencing analysis of this short transcript showed exon 13 skipping. C: control. Pt: patient. (C) This aberrant splicing is predicted to result in Tyr538Arg fs*3.

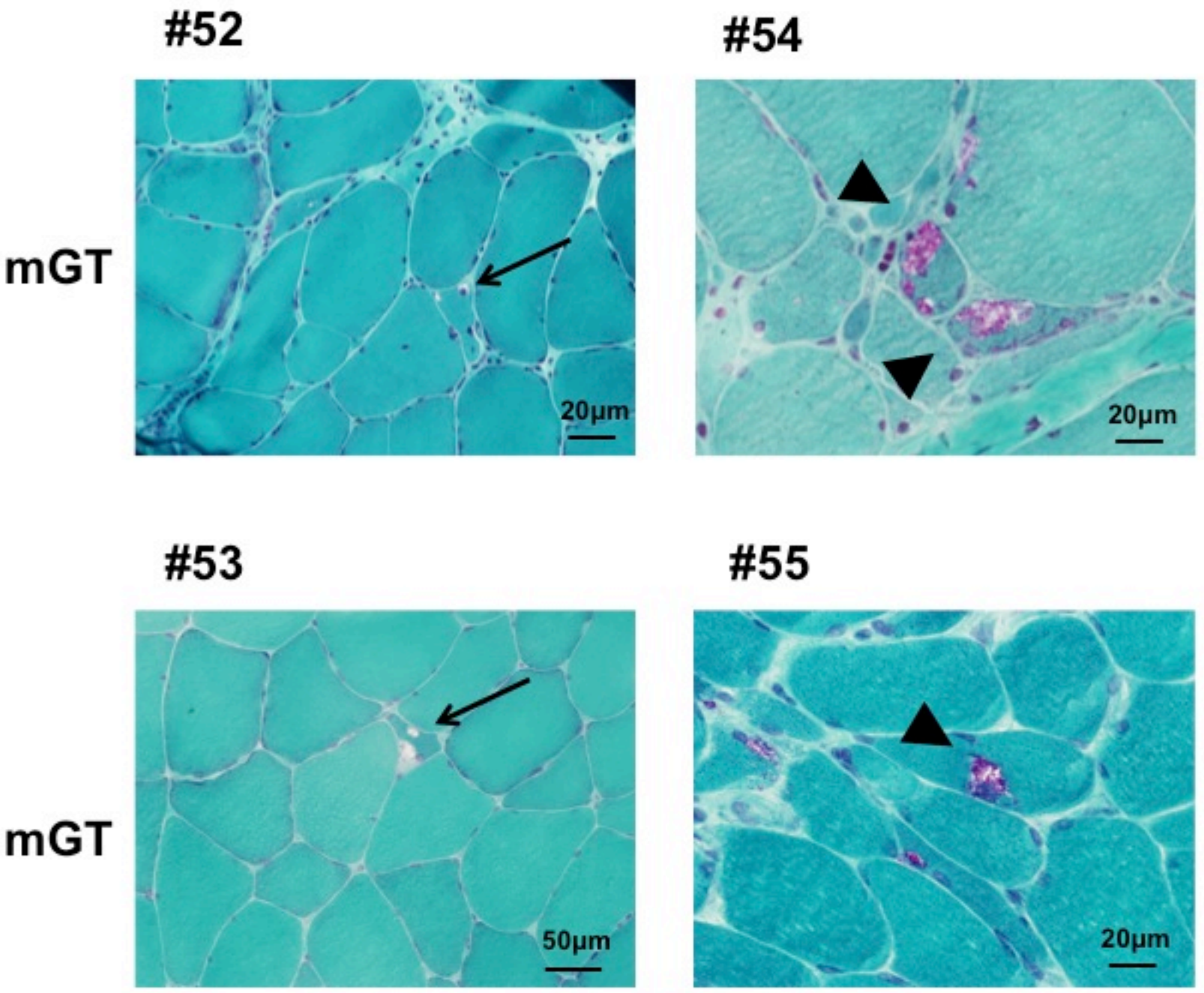


Supplemental Figure 28
HE staining showed only nonspecific changes with variation in fiber size in patients with *PYGM* mutations (#49, #50). PAS staining was unremarkable. Phosphorylase staining in both patients was absent, which is compatible with McArdle disease.

#51



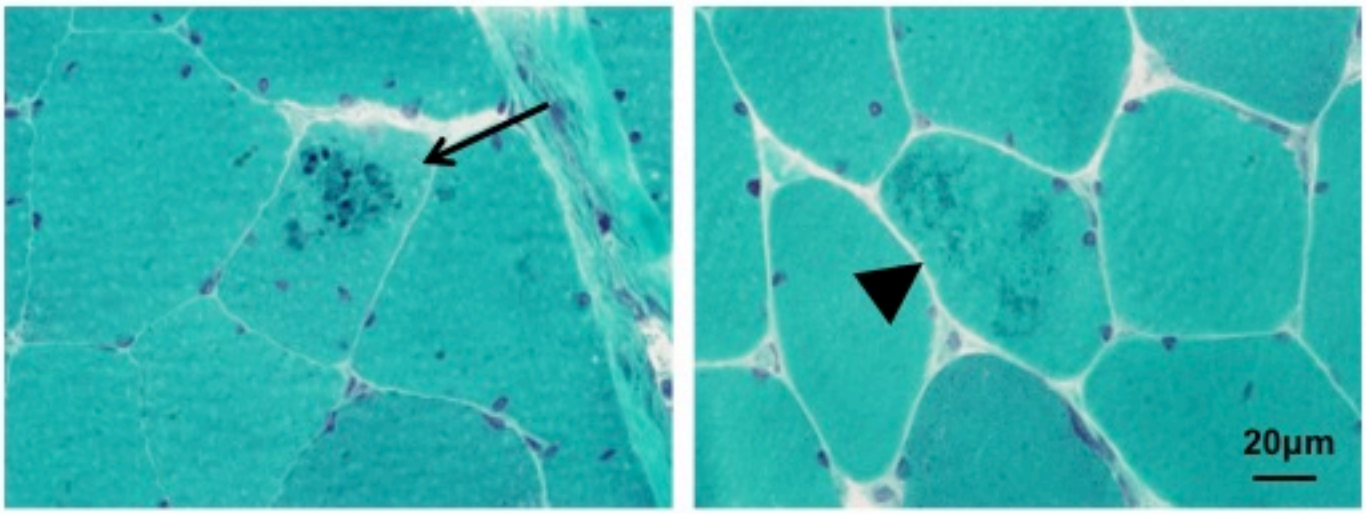
Supplemental Figure 29
Modified Goromi-trichrome staining of a patient' muscle with *DNAJB6* mutation (#51) showed occasional fibers with spheroid bodies.



Supplemental Figure 30
Modified Gomori-trichrome staining of GNE myopathy patients' muscle. In two patients (#52, #53), rimmed-vacuoles, a hallmark of this disease, were not prominent (arrow). In patients #54 and #55, rimmed vacuoles were prominent (arrow head).

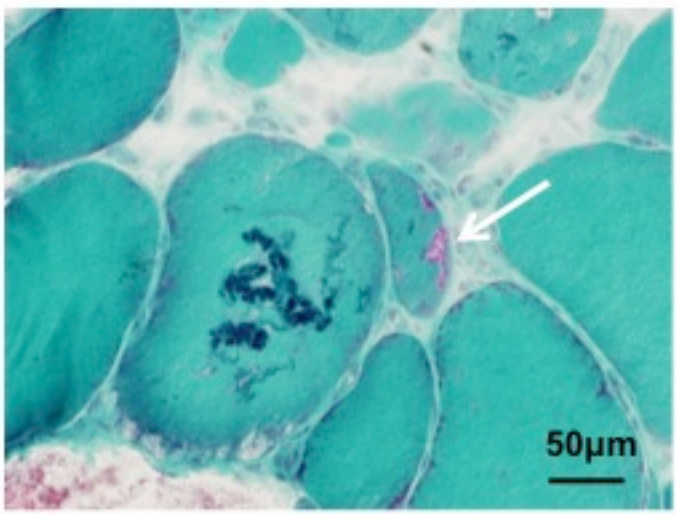
#56

mGT



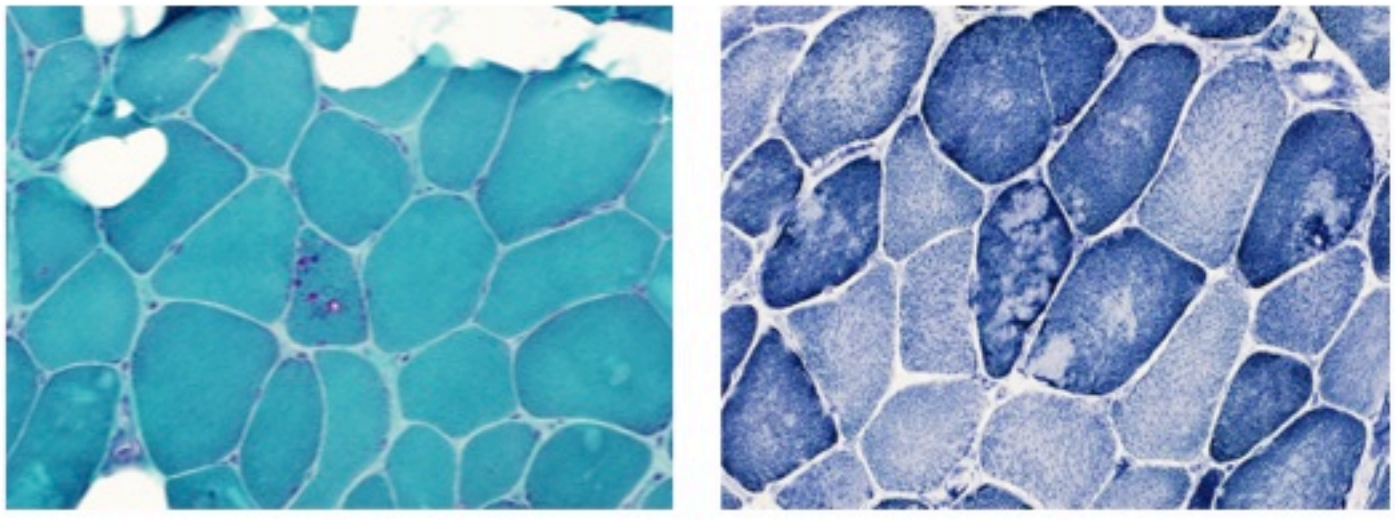
#57

mGT



Supplemental Figure 31
Patient #56 harbored mutation in *MYH2* exhibited some fibers with cytoplasmic bodies (arrow) and spheroid bodies (arrow head) in modified Gomori-trichrome staining. In patient #57 with *MYOT* mutation, fibers with rimmed vacuoles were also seen (white arrow).

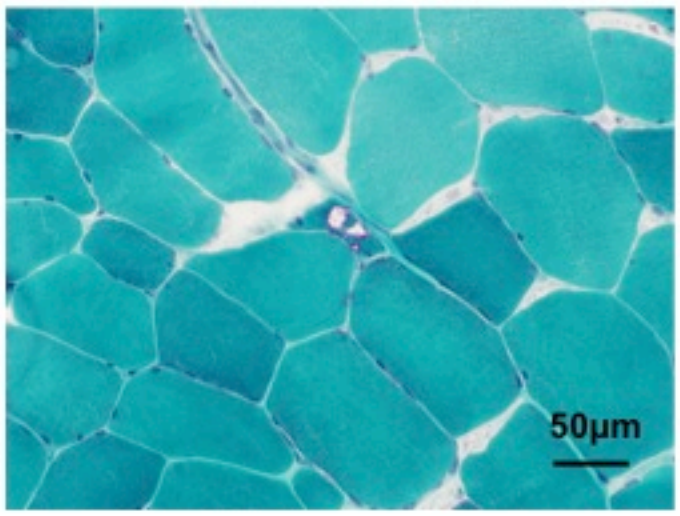
#58



Supplemental Figure 32
Patient with *SEPN1* mutations (#58). On modified Gomori-trichrome staining (left), fibers with rimmed-vacuoles were seen. On NADH staining (right), numerous fibers with multi-minicore or moth-eaten fibers were seen.

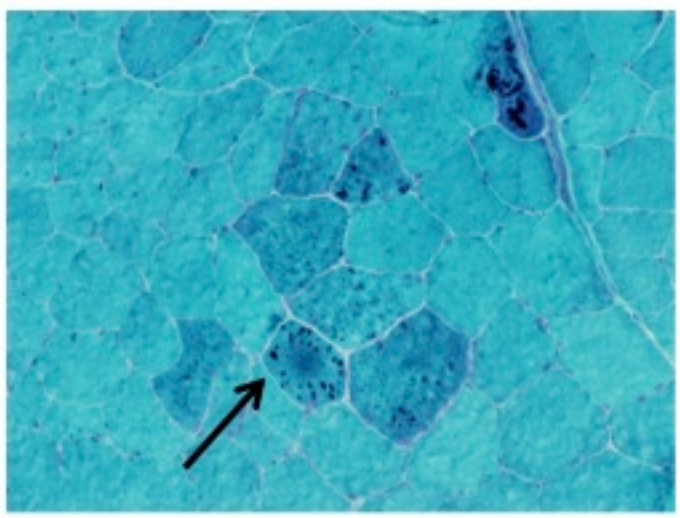
#59

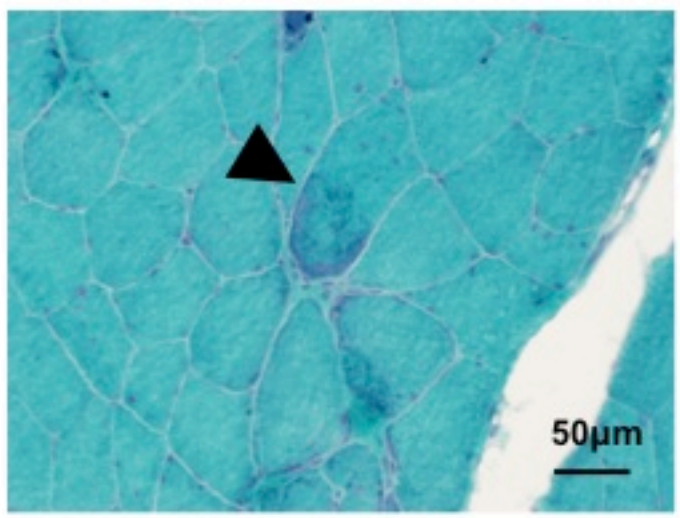
mGT



#60

mGT

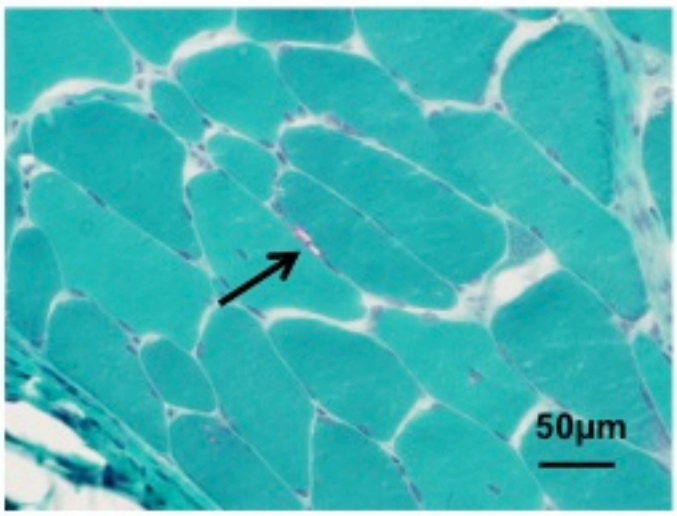




Supplemental Figure 33
On modified Gomori-trichrome staining, no cytoplasmic bodies were seen in patient #59 with *TTN* mutation, but fibers with rimmed vacuoles were detected. In patient #60, typical necklace bodies (arrow) and spheroid bodies (arrowhead) were seen.

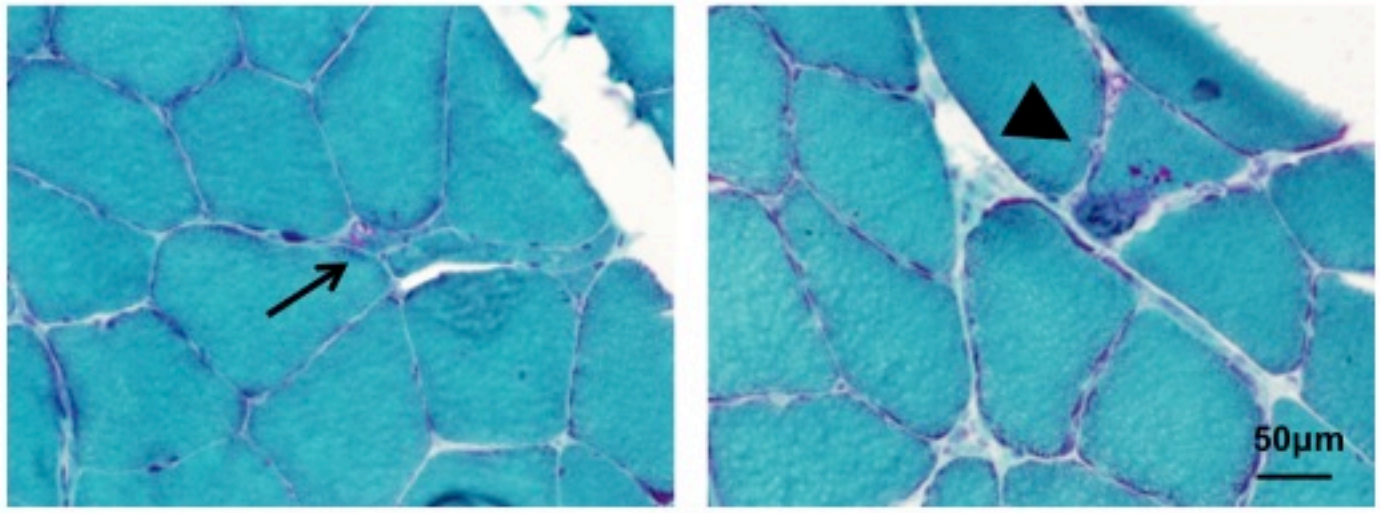
#61

mGT

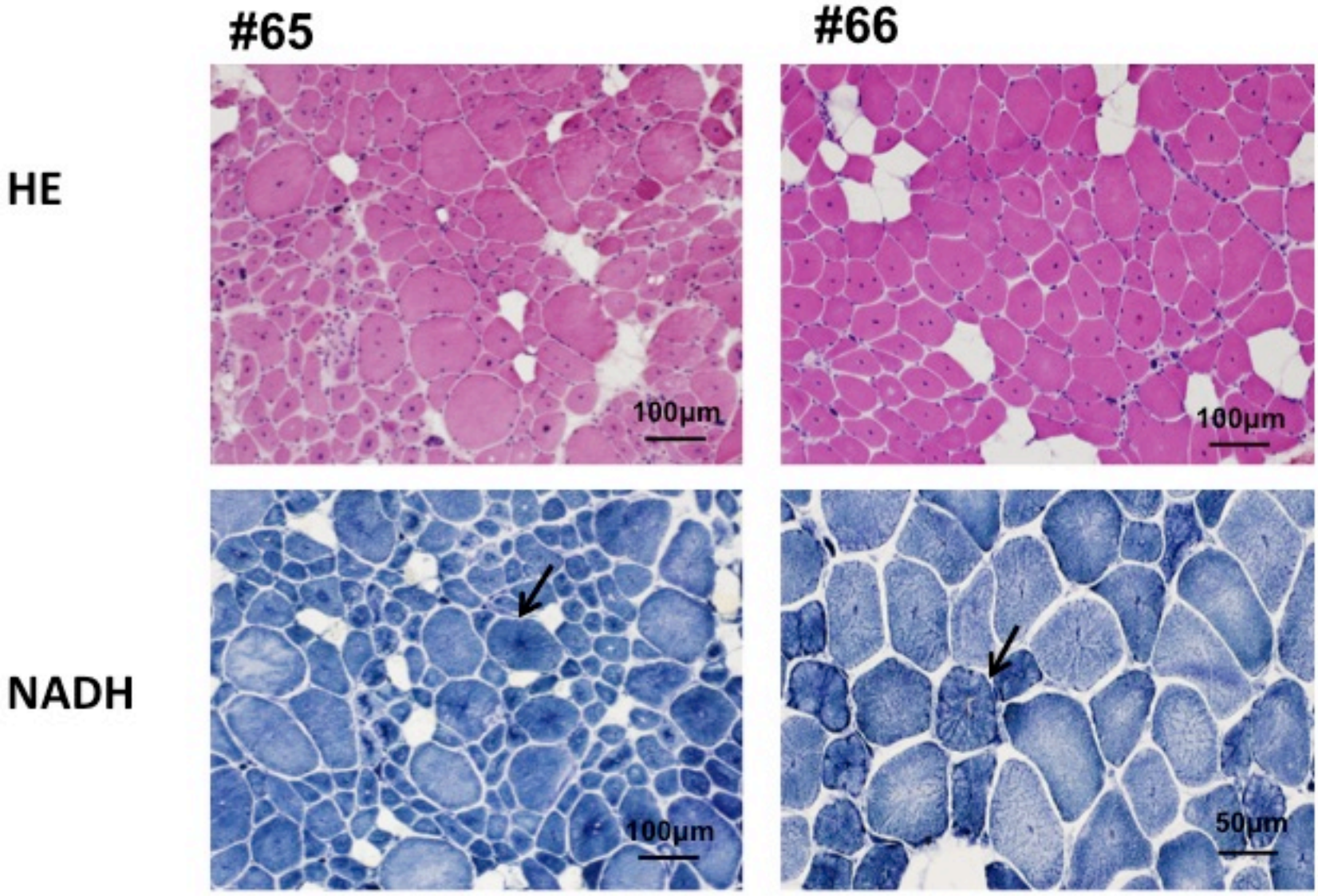


#62

mGT

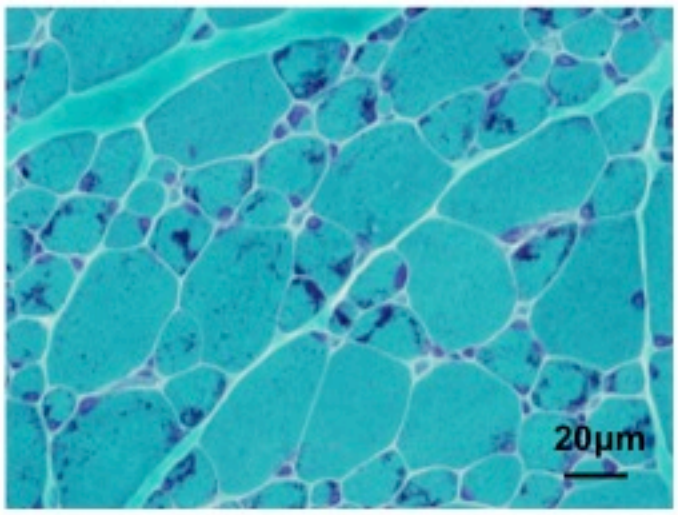


Supplemental Figure 34
Modified Gomori-trichrome staining of samples from both patients with *VCP* mutations (#61, #62) showed fibers with rimmed vacuoles (arrow). In patient #62, fibers with cytoplasmic bodies were also seen (arrowhead).

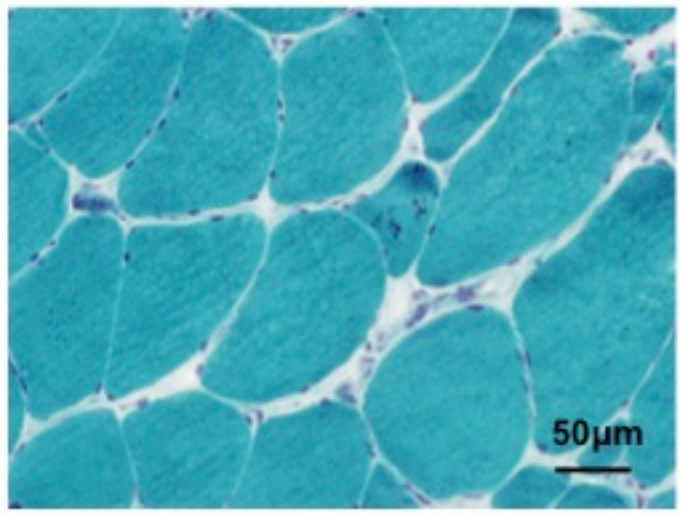


Supplemental Figure 35
Patients #65 and #66 had *DNM2* variants of unknown significance. HE staining showed variation in fiber size and increased number of fibers with internalized nuclei. NAHD staining showed radiated strands (arrow).

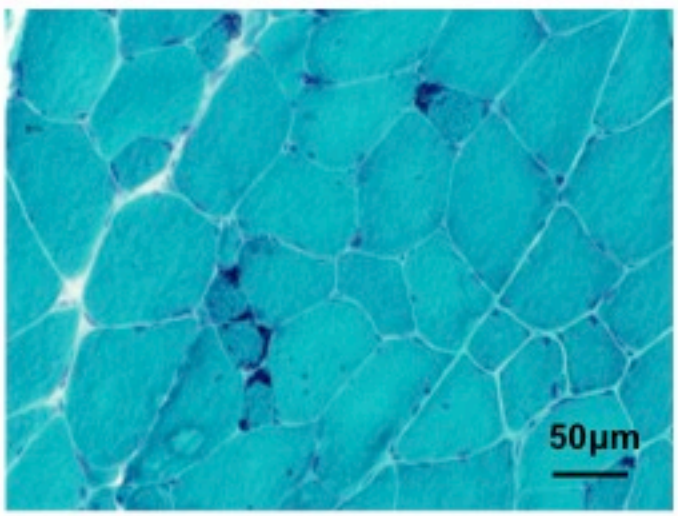
#67



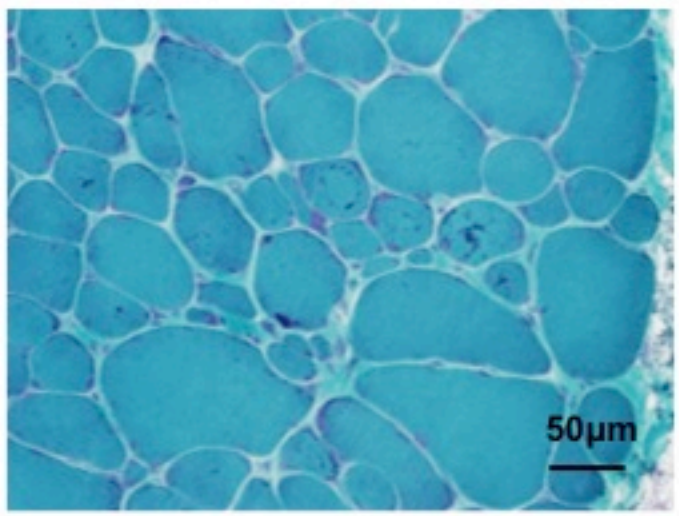
#70



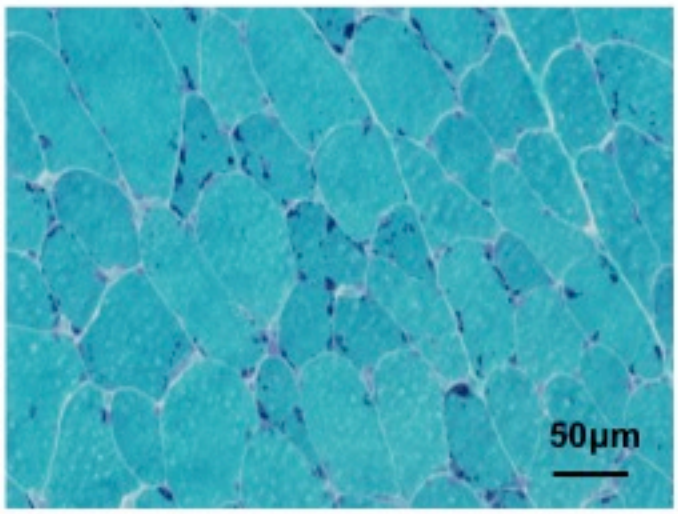
#68



#71



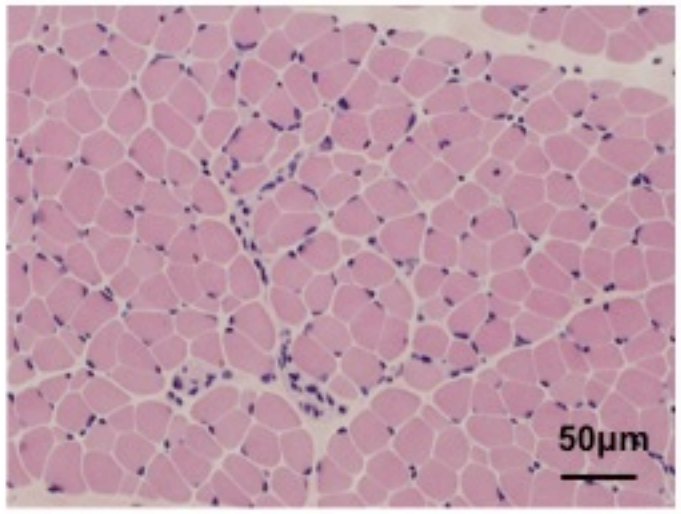
#69



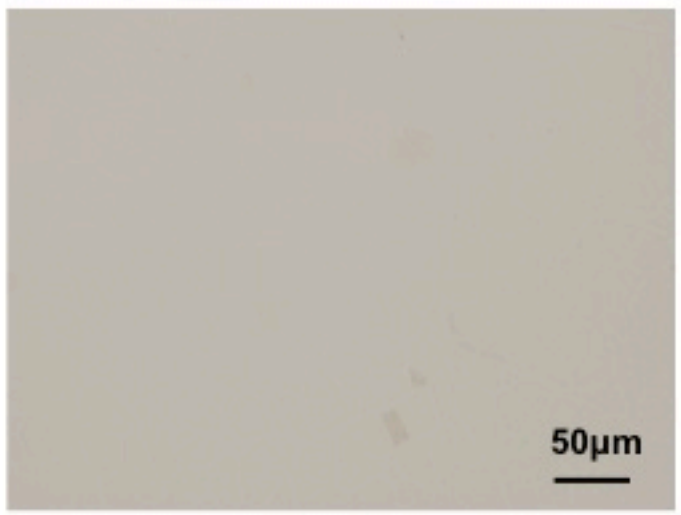
Supplemental Figure 36
Patients with *NEB* variants suspected to be pathogenic (#67-71). Modified Gomori-trichrome staining showed some fibers with nemaline bodies. Nemaline bodies were especially seen in type 1 fibers.

#72

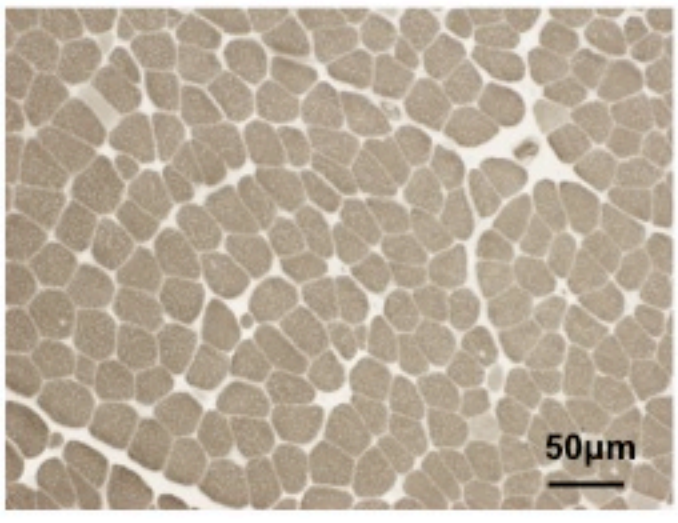
HE



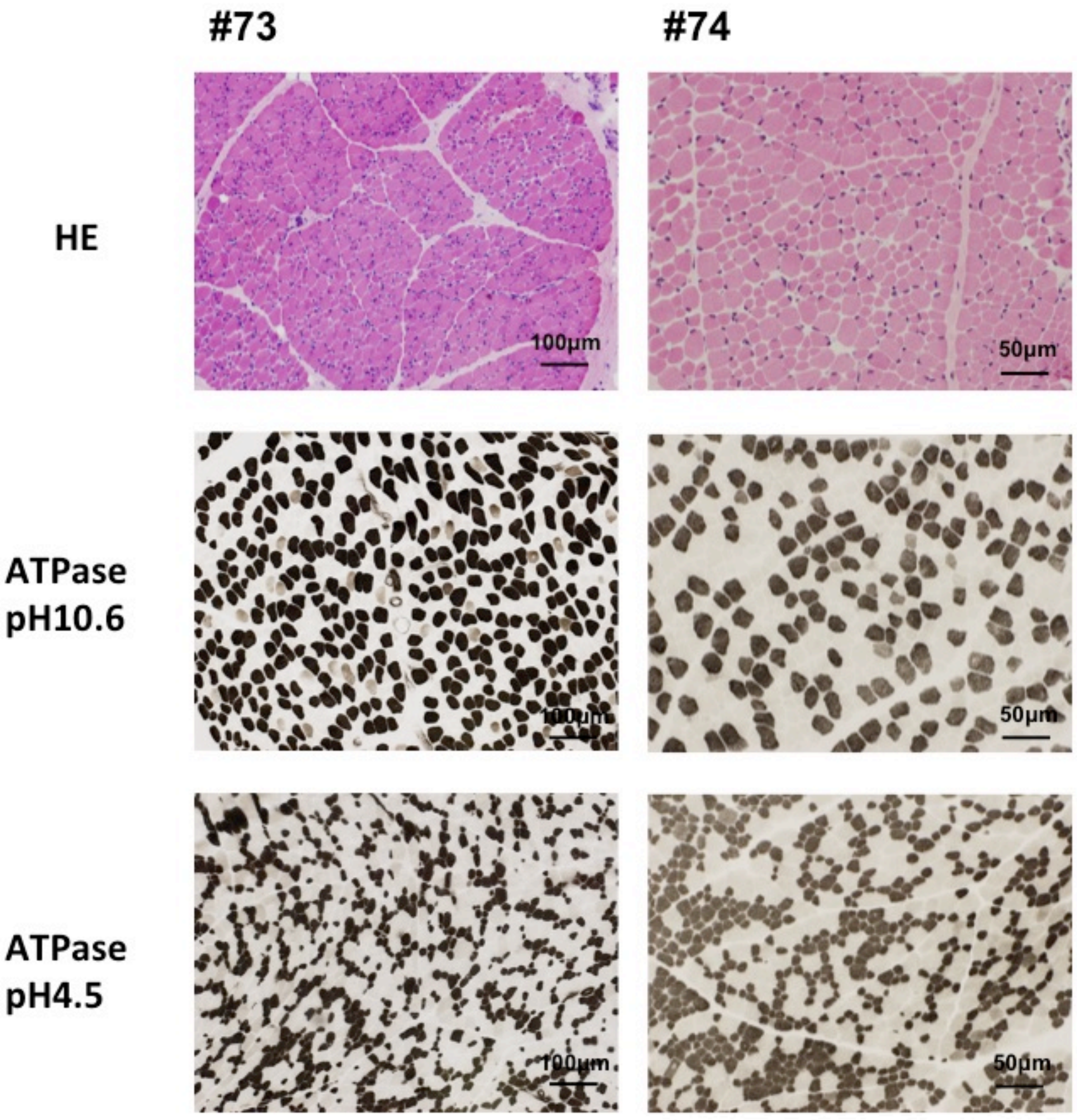
ATPase
pH10.6



ATPase
pH4.5



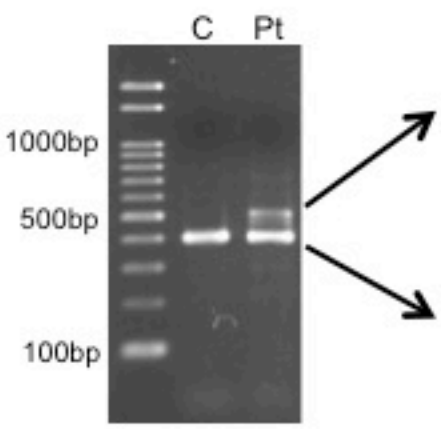
Supplemental Figure 37
No muscle fibers were stained on myosin ATPase staining at pH 10.6 in patient #72, suggesting uniform type 1.



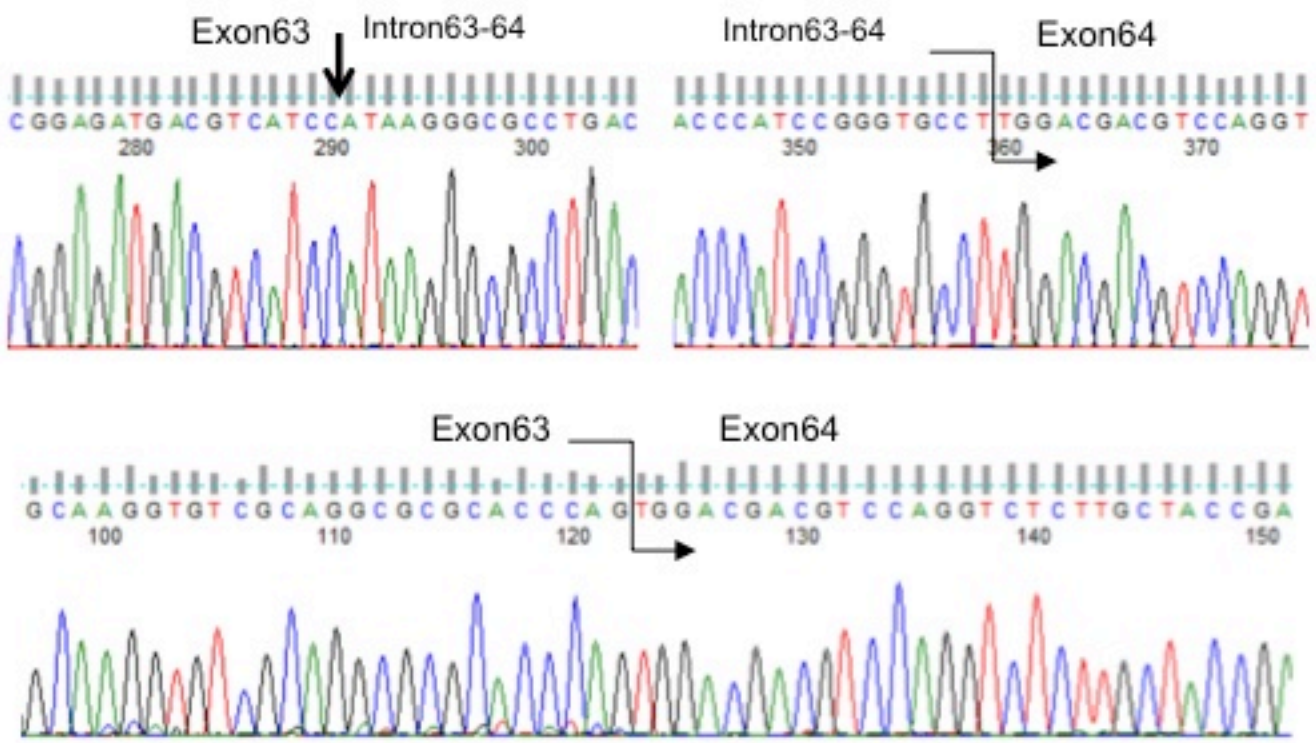
Supplemental Figure 38
Patients with *RYR1* variants (#73, #74) suspected to cause CFTD. Myosin ATPase staining showed type1 fibers were smaller than type 2 fibers.

#73

A

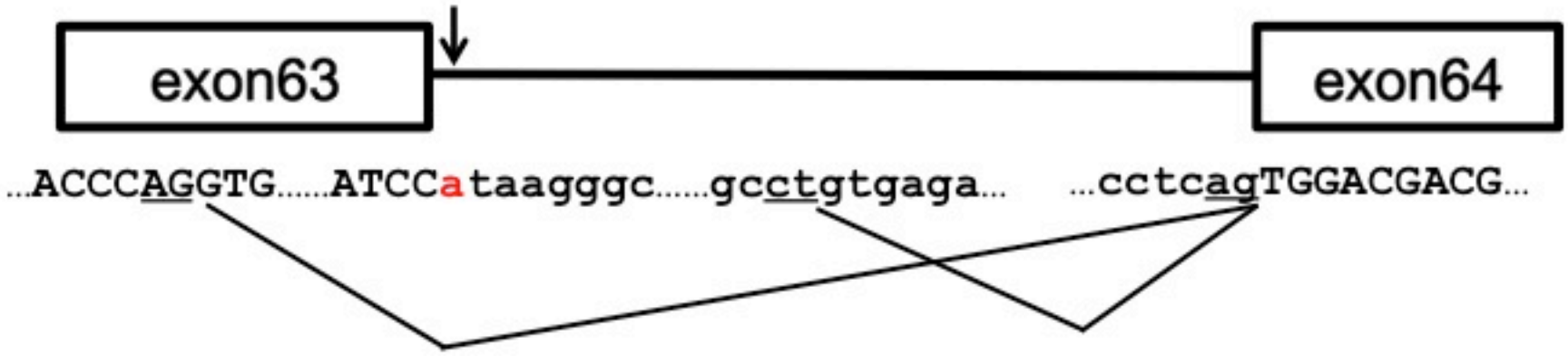


B



C

RYR1:c.9472+1G>A

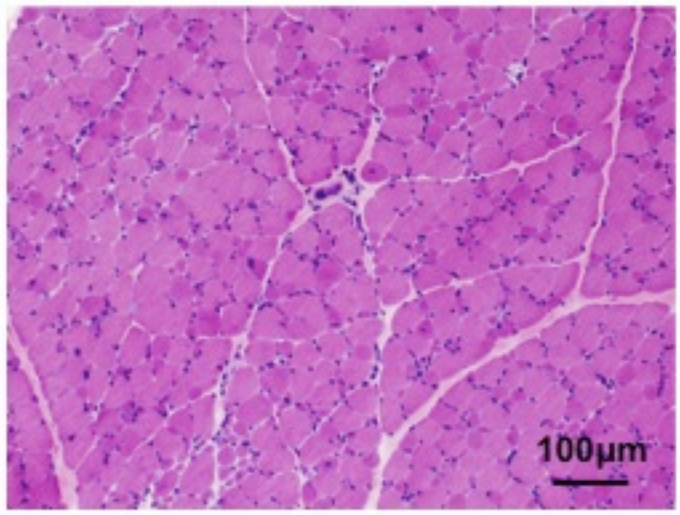


cDNA	c. 9364_9472del	c. 9472_9473insATAAGG GCGCCTGACCCAAGGGCAGGTTGCG GGGAGTCAGTGTGGCCAACACCACCC ATCCGGGTGCCT
protein	p.(Val3122Trp fs*53)	p.(Leu3158His fs*5)

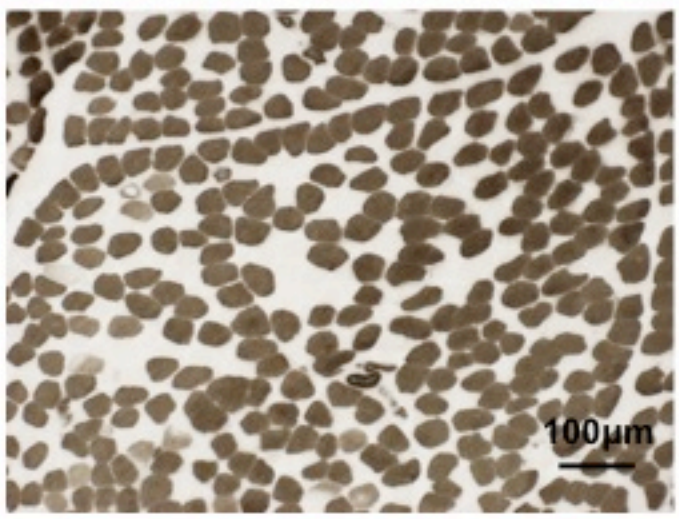
Supplemental Figure 39
Consequence of c.9472+1G>A in *RYR1* transcripts in patient #73. (A) Muscle derived cDNA was amplified using primers at exon 63 and 65. PCR products were sequenced. C: control. Pt: patient. (B-C) Two aberrant splicing variants were detected, both of which caused frameshift. Normal transcript was also detected (not shown).

#75

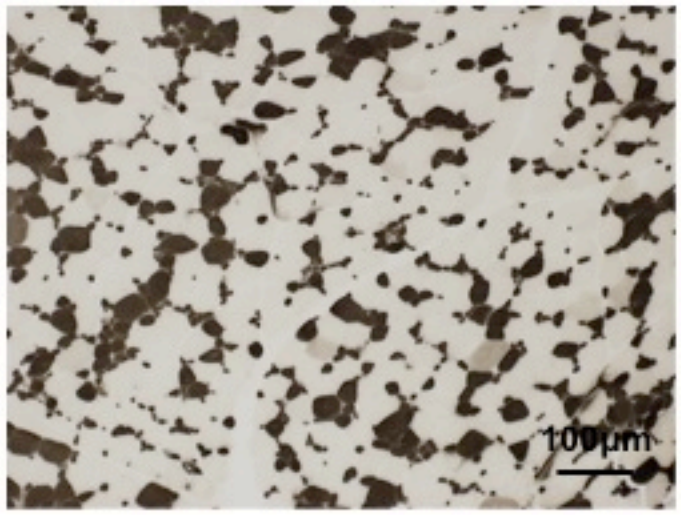
HE



ATPase
pH10.6

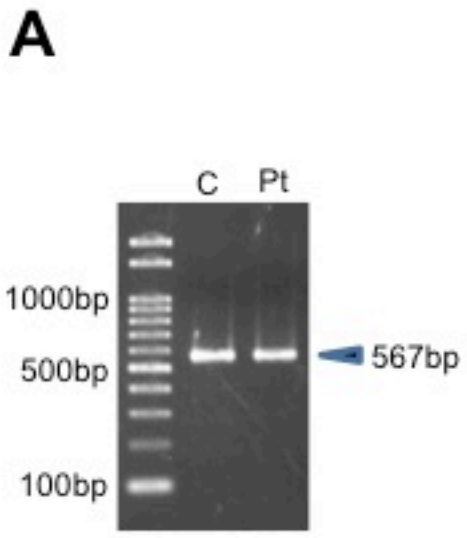


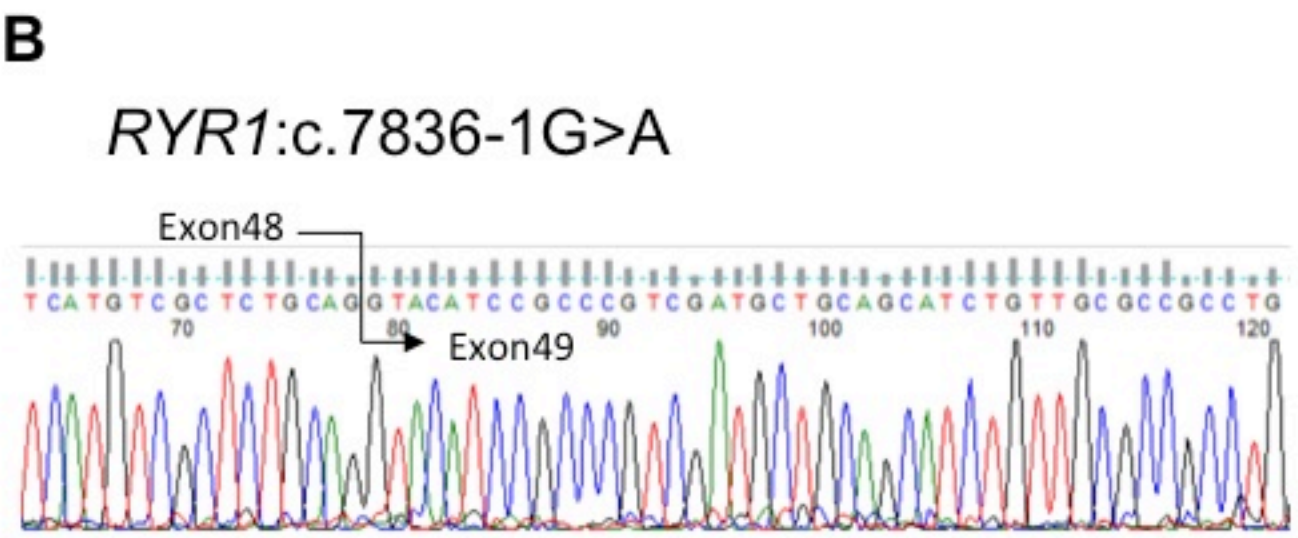
ATPase
pH4.5

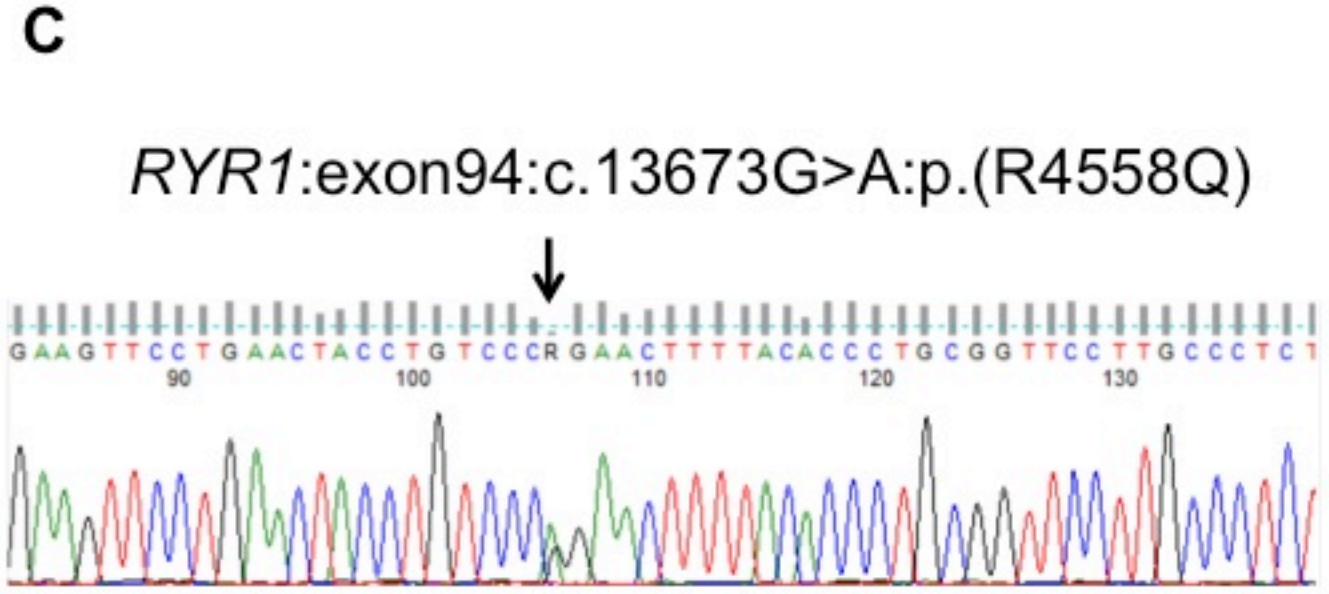


Supplemental Figure 40
Patient with *RYR1* variants suspected to cause CFTD (#75). Myosin ATPase staining showed type1 fibers are smaller than other fibers.

#75





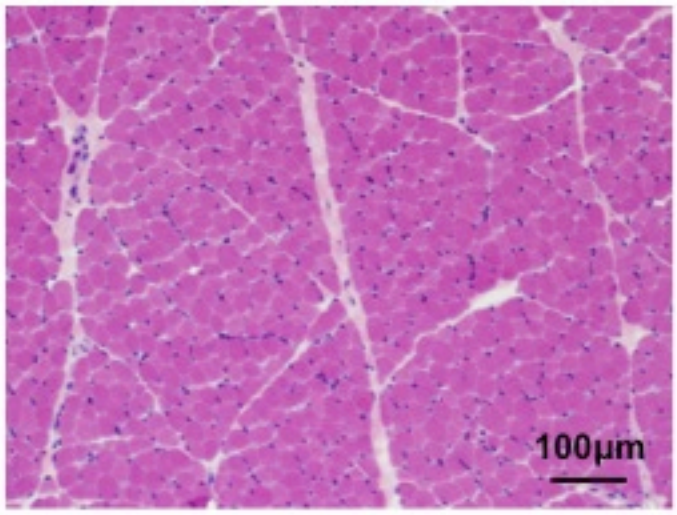


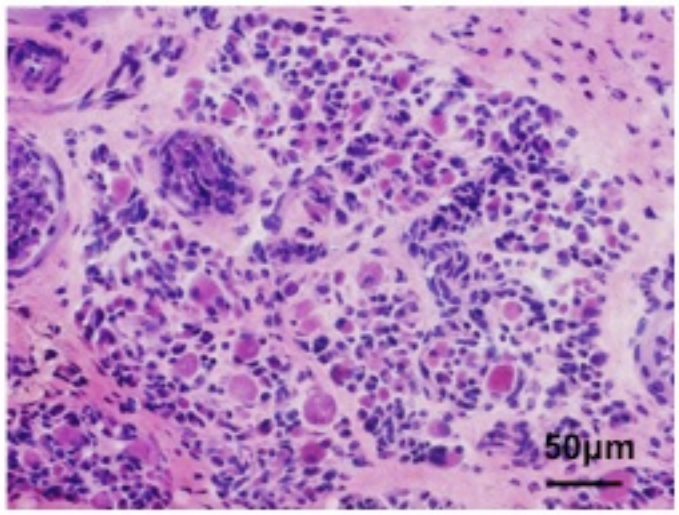
Supplemental Figure 41
Consequence of c.7836-1G>A in *RYR1* in patient #75. (A) Muscle derived cDNA was amplified using primer at exon 48 and 52 showing single band. C: control. Pt: patient. (B) Exon 48 and Exon 49 were normally spliced. (C) Transcripts of c. 1367G>A-harboring and wild-type allele (arrow).

#76

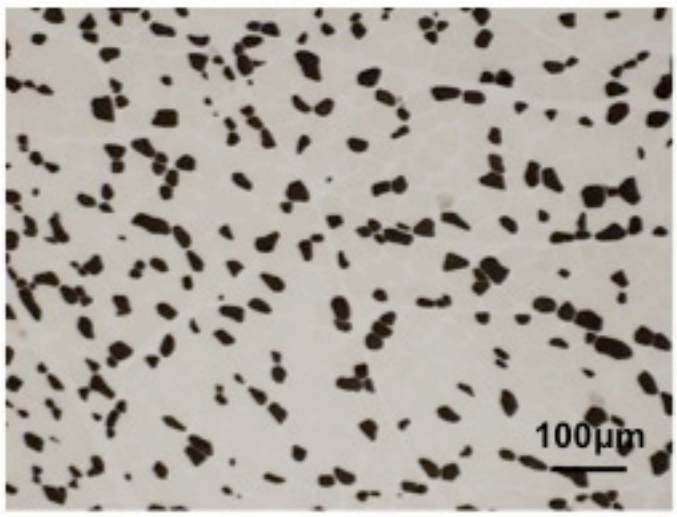
#77

HE

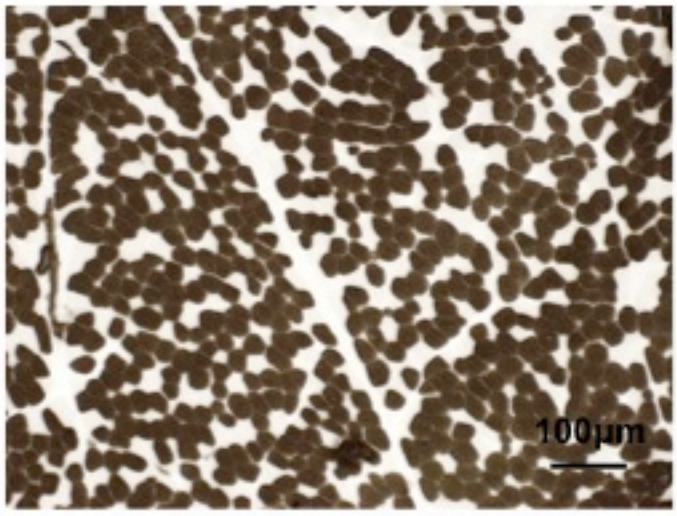




ATPase
pH10.6



ATPase
pH4.5



Supplemental Figure 42
Patients #76 with *RYR1* variants suspected to cause type 1 fiber predominance. Myosin ATPase staining showed type1 fibers were smaller than other fibers. Patient #77 possessed extremely small muscle fibers as shown in the HE staining images.