

ORIGINAL ARTICLE

Novel pathogenic variants and genes for myopathies identified by whole exome sequencing

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Introduction

Myopathies and muscular dystrophies can be classified into a large heterogeneous subgroup of neuromuscular

Abstract

Neuromuscular diseases (NMD) account for a significant proportion of infant and childhood mortality and devastating chronic disease. Determining the specific diagnosis of NMD is challenging due to thousands of unique or rare genetic variants that result in overlapping phenotypes. We present four unique childhood myopathy cases characterized by relatively mild muscle weakness, slowly progressing course, mildly elevated creatine phosphokinase (CPK), and contractures. We also present two additional cases characterized by severe pre-natal/neonatal myopathy. Prior extensive genetic testing and histology of these cases did not reveal the genetic etiology of disease. Here, we applied whole exome sequencing (WES) and bioinformatics to identify likely causal pathogenic variants in each pedigree. In two cases, we identified novel pathogenic variants in *COL6A3*. In a third case, we identified novel likely pathogenic variants in *COL6A6* and *COL6A3*. We identified a novel splice variant in *EMD* in a fourth case. Finally, we classify two cases as calcium channelopathies with identification of novel pathogenic variants in *RYR1* and *CACNA1S*. These are the first cases of myopathies reported to be caused by variants in *COL6A6* and *CACNA1S*. Our results demonstrate the utility and genetic diagnostic value of WES in the broad class of NMD phenotypes.

diseases (NMDs) that are primarily associated with dysfunction of muscle fibers. Identifying the genetic cause of myopathies can be challenging as symptoms overlap and numerous genetic defects in many genes may underlie the

clinical pathology of disease. While symptoms can direct successful genetic diagnosis through testing of single genes or small panels of genes, they may also lead to costly, time-consuming, and often unsuccessful attempts at genetic diagnosis. Next-generation sequencing (NGS) can greatly improve the ability to identify pathogenic variants with a single, timely, affordable assay and is beginning to revolutionize genetic testing (Ng *et al.* 2009, 2010). We thus applied WES to congenital and childhood genetic diagnostic odyssey cases of myopathy/muscular dystrophy (MD). We provide a clinical description of six cases (Table 1) and describe the candidate pathogenic variants identified in each (Table 2). We first present three cases with Collagen 6 (Col6) myopathies which all carry an identical pathogenic variant in *COL6A3* (OMIM# 120250). Importantly, we provide evidence that variants in *COL6A6* likely result in myopathy. Next, we present a case with a phenotype very similar to two of the Col6 myopathy cases, but instead was found to have Emery–Dreifuss Muscular Dystrophy (EDMD) caused by a novel variant in *EMD* (OMIM# 300384) at a known pathogenic genomic position. Finally, we present two cases with calcium channelopathies. We present a dominant case of central core disease (CCD) caused by an insertion in *RYR1* (OMIM# 180901), followed by evidence for the first *CACNA1S* (OMIM# 114208)-related congenital myopathy with ophthalmoplegia. Our results shed light on the genetic etiology of several related myopathy cases and provide evidence for the effectiveness of WES in aiding in resolving the genetic diagnosis.

Materials and Methods

Patient recruitment and sample collection

Children with clinically diagnosed myopathies and relevant family members were recruited and consented for participation in our research study according to our current Western Institutional Review Board approved protocol (#20120951). Whole blood samples were sent directly to the Dorrance Clinical Laboratory at the Translational Genomics Research Institute (TGen), a CLIA-certified laboratory. DNA was isolated from whole blood and quality and quantity were determined by NanoDrop (ThermoFisher Scientific, Waltham, MA) and Qubit DNA (Life Technologies, Grand Island, NY) assays.

Whole exome library preparation and sequencing

Whole genome (WG) libraries were prepared according to the manufacturer's protocols using a TruSeq Library Preparation kit (Illumina, San Diego, CA) or a Library

Preparation kit (Kapa Biosystems, Wilmington, MA). DNA quantity and quality were determined by analysis on a High Sensitivity DNA Bioanalyzer chip (Agilent Technologies, Santa Clara, CA). Double-stranded DNA concentrations were obtained using a Qubit Hi-sensitivity DNA assay kit (Life Technologies). WG libraries were pooled and exome enrichment was performed using a modified TruSeq Exome Enrichment kit (Illumina) protocol, where all amplification of exome-enriched libraries was performed using the Kapa Biosystems' library amplification kit. The enriched libraries were quantitated and qualified using Qubit and Bioanalyzer chip assays as described above. The enriched exome libraries were clustered using the Illumina cBot. Sequencing was performed on Illumina HiSeq 2000/2500 systems using Illumina SBS Kit v3 with 83 × 83 or 100 × 100 base pair (bp) paired-end reads with single index reads of 7 bp according to the manufacturer's protocols.

Bioinformatic analysis

Raw sequence data were converted to FASTQ files using Illumina's BCL Converter tool which were aligned to build 37 of the human reference genome (<http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/>) using the Burrows-Wheeler Alignment tool (BWA) (Li and Durbin 2009) and sorted with SAMtools (Li *et al.* 2009) to create binary sequence (BAM) files. PCR duplicates were flagged for removal using Picard (<http://picard.sourceforge.net>), which was also used to evaluate other metrics such as coverage and GC metrics. Figure S1 displays basic sequencing coverage metrics.

Variants were called and BAM files were insertion/deletion realigned and recalibrated using The Genome Analysis Toolkit (GATK) (McKenna *et al.* 2010). The variant call files were annotated with information from the Genetic variant annotation and effect prediction toolbox (SnPEff) (Cingolani *et al.* 2012), then further annotated with a custom in-house annotation interface tool with information from numerous databases such as ClinVar (www.ncbi.nlm.nih.gov/clinvar/), Polyphen-2 (Adzhubei *et al.* 2010), FATHMM (Shihab *et al.* 2013), SIFT (Kumar *et al.* 2009), Clinical Genomic Database (Solomon *et al.* 2013), and The National Heart, Lung, and Blood Institute's GO Exome Sequencing Project (ESP) (ESP 2013). Allele counts from ExAC (Exome Aggregation Consortium 2014) and CADD (Kircher *et al.* 2014) scores were also obtained. Variants were custom sorted for each family based on inheritance patterns and by annotations. Suspect variants classified as "likely pathogenic" or "pathogenic" based on ACMG guidelines (Richards *et al.* 2008) were clinically confirmed by Sanger sequencing at GeneDX (Cases 2–6) or to ARUP Laboratories (Case 1).

Table 1. Case phenotype summary.

Family	F038	F049
Gender	Male	Male
Ethnicity	Caucasian	Caucasian
Birth	G2, P1; no problems other than congenital club feet	G2, P1; 40 weeks; spontaneous vaginal; APGAR normal
Age of onset (retrospective)	Congenital	~1 year
Age of first related clinical visit	Early childhood	11 years
Age at time of study	17 years	12 years
Inheritance	Autosomal recessive	Autosomal recessive
Disease gene	COL6A3	COL6A3
Diagnosis based on genetic findings (OMIM#)	Bethlem myopathy (158810)	Bethlem myopathy (158810)
Contractures/joints	Hamstrings, ankles, scoliosis, overlapping toes, congenital club feet	Hamstrings, ankles, some scapular winging, very slight pectus excavatum
Muscle biopsy histopathology and EM	H&E revealed fiber size variation, split fibers, internal nuclei, separation of fibers, and connective tissue and endomysium proliferation. ATPase demonstrated moderate degree of fiber type grouping. NADH staining revealed loss of honeycomb fibers and abnormal myofibrillar architecture. Fiber type changes suggestive of myopathy. Normal expression of dystrophin (Rod, c-terminal, and n-terminal domains), SGC(α , β , δ , γ), spectrin, dysferlin, and α -dystroglycan by IHC. Normal ADD, COX, and SDH, enzymatic activity staining. No ragged red fibers detected by trichrome staining	Vastus lateralis muscle biopsy: H&E and ATPase staining revealed marked fiber size variation with large hypertrophic and tiny multinucleated atrophic fibers. Majority of fibers show central nuclei and occasional fiber splitting. Scattered regenerating and degenerating fibers with mild inflammation. Markedly increased connective and adipose tissue by H&E and trichrome. NADH revealed slight mottling of fibers with paler centers. Normal lipid and glycogen content by Oil-red-O and PAS. Normal COX and SDH enzymatic activity staining. Dystrophic changes without identifiable loss of sarcoplasmic membrane proteins including myosin fast, myosin slow, dystrophin 1-3, spectrin, merosin, laminin-2, LAMP2, adhalin, sarcoglycan (γ , β , δ), dystroglycan (α , β), dysferlin, Collagen 6. EM demonstrates well-formed sarcomeres with some focal Z-band streaming (one sarcomere length), small degenerated and atrophic fibers show loss of filaments but no inclusions. Normal number and morphology of mitochondria
CPK (international units)	600–1000	500–700
Milestone delays	walk – 14 months	walk – 15 months
Musculature changes	Mild wasting of rectus femoris, mild calf hypertrophy	Mild atrophy of scapular muscles, no obvious muscle atrophy in his anterior or posterior quadrant of his lower extremities
Descriptions of muscle weakness	Bilateral; deltoids (5), deltoids (4-), hip flexors (4-), hip extensors (4-), foot dorsiflexors (4-), neck flexor (5), neck extensor (5), biceps (5), triceps (minor detectable weakness), wrist flexors (5), wrist extensors (5)	Predominantly symmetric; abdominals (3+), supraspinatus (4), deltoids (4-), shoulder girdle muscles (4-), biceps (4+), triceps (5-), wrist flexors (5), wrist extension (5-), grasp (4), hip abduction (3+), hip adduction (5-), hip flexion (4), hip extension (4+), knee extension (4), knee flexion (4+), foot dorsiflexion (R4, L4-) Ankle dorsiflexion (3+)
Ophthalmoplegia, ptosis, nystagmus, facial diplegia	No ophthalmoplegia, no nystagmus, no ptosis	No ophthalmoplegia, no nystagmus, no ptosis
Fatigue	Excessive fatigue, especially afternoon	Easily fatigued, especially afternoon
Deep knee bend test	Age 15 difficulty performing, age 17 able to do several	Unable to do repetitive deep knee bends
Toe walk test	Able	Able, toe walker
Heel walk test	Unable	Unable
Gowers' test	Difficulty rising from floor	Not Gowers' but not quite normal
Gait	Abnormal gait with mild waddling quality	Always had gait difficulties, had history of frequent falls, gait is lordotic, mild waddling

(Continued)

Table 1. Continued.

Family	F038	F049
Choreiform/spasticity/tremor	None	Possible mild tremor, family history of tremor
Deep tendon reflexes	Normal	Normal
EMG	Normal peripheral motor and sensory nerve conduction with abnormal spontaneous activity, chronic motor neuropathy	Left anterior tibialis: 1+ fibrillations, and 2+ positive sharp waves, significant 3+ polyphagia and early recruitment pattern
Cognition and speech	Normal, above average	Normal, history of anxiety
Dysmorphology	None, very thin	None
Respiration	Regular, unlabored, normal FVC and FEV1, many lung infections as infant	Regular, unlabored, sighs occasionally, normal FVC and FEV1
Pulse/blood pressure	Regular, normal	Regular, normal
EKG	Normal	Normal on multiple occasions
GI	History of nausea and vomiting, GI trouble, but GI study normal, mostly resolved	None
Skin	Normal	Normal
Prior negative testing	Normal genetic sequencing: <i>SMN1, FKR, MYOT, LMNA, CAV3, CAPN3, SGCA, SGCB, SGCD, SGCG, TCAP, TRIM32, PMP22, GJB1, MPZ, EGR2, NEFL, PRX, GDAP1, LITAF, MFN2</i>	Normal genetic sequencing: <i>ANOS, SMN1, FSHD, CAPN3, FKR, LMNA, TCAP, DYSF, TRIM32, SGCA, SGCB, SGCD, SGCE, SGCG, TTN</i> (partial)
Other notes of interest	Age 5–6 period of significant unexplained weight loss, BMI consistently less than 1st centile, malnourished appearance, difficulty gaining weight; no significant sleep-disordered breathing, but some sleep disturbances	Vitamin D insufficiency; age 11–12 years; period of 5 kg unexplained weight loss; borderline Arnold–Chiari malformation type 1 (considered unrelated and benign)

Family	F041	F043
Gender	Female	Male
Ethnicity	Caucasian	Caucasian
Birth	35 weeks; emergency C-section; not breathing (resuscitated)	G2, P0; 42 weeks; vaginal, vacuum assist; tight nuchal cord; APGAR 5, 9; respiratory distress with resuscitation
Age of onset (retrospective)	Prenatal	~1 year
Age of first related clinical visit	Prenatal	6 years
Age at time of study	9 years	12 years
Inheritance	Autosomal Recessive, possibly complex recessive	X-Linked Recessive
Disease Gene	<i>COL6A6</i> <i>COL6A3</i>	<i>EMD</i>
Diagnosis based on genetic findings (OMIM#)	Collagen 6 myopathy	Emery–Dreifuss muscular dystrophy 1, X-linked (310300)
Contractures/joints	Fingers, wrist, elbow, shoulder, ankles, knees, hips – improved over time. Arthrogryposis. Hyperextensions at her knees and at her left elbow. When seated her feet are extended at ankles. Excessive lordosis and mild lumbar scoliosis	Scapular winging, elbows, hamstring, ankle, pes cavus, lordosis. Contractures on left are more severe.
Muscle biopsy histopathology and EM	No record of muscle biopsy	H&E and ATPase (pH 4.6 and 9.6) and NADH staining revealed mild myopathic changes with no fibrosis or inflammation. Modest number of scattered moderately atrophic fibers that tend to be rounded or angulated. Slight fiber type grouping was noted. Normal IHC for dystrophin (rod, C-terminus, and N-terminus domains), sarcoglycan($\alpha, \beta, \delta, \gamma$), dysferlin, alpha dystroglycan, and calpain. Nonspecific esterase reaction demonstrated rare nonspecific esterase-positive atrophic fiber. Normal COX and SDH enzymatic activity staining. Normal lipid and glycogen content by Oil-red-O and PAS. Normal connective tissue.

(Continued)

Table 1. Continued.

Family	F041	F043
CPK (international units)	No information	500–800
Milestone delays	Hold up head 6 months sit up 6 months roll over 6 months reach/grab 9 months stand 2 years crawl 3 years walk 5 years	Sit up 7 months roll over 4 months crawl 8 months stand 12 months walk 14 months
Musculature changes	No information	No muscle wasting noted
Descriptions of muscle weakness	Dysphagia, dysarthria, generalized weakness	Deltoids (4+), biceps (5), triceps (5), wrist flexors (5), wrist extensors(5), finger flexors(5), iliopsoas (4), hip flexor (3) and extensor (4-), foot dorsiflexors (3). Mother described left side as weaker
Ophthalmoplegia, ptosis, nystagmus, facial diplegia	No information	No ophthalmoplegia, no nystagmus, no ptosis
Fatigue	No information	Easily fatigued
Deep knee bend test	No information	Fatigue after 4 deep knee bends
Toe walk test	No information	Able, toe walker
Heel walk test	No information	Unable
Gowers' test	No information	Modified Gowers'
Gait	In walking, her feet turn out to the side and she tends to lead with her right leg keeping her left knee straight	Abnormal gait with mild waddling quality
Choreiform/spasticity/tremor	No information	None
Deep tendon reflexes	No information	Decreased but obtainable
EMG	No information	Abnormal motor unit analysis, no peripheral neuropathy
Cognition and speech	Significant expressive language disorder, very limited speech – uses single word utterance most of the time. No cognitive defects.	Normal
Dysmorphology	Significant craniofacial abnormalities; dolichocephaly, obligatory open-mouth posture with significant dental crowding, micrognathia, narrow mandible, elevated palate. Does not achieve mouth closure; persistent drooling	None
Respiration	Not breathing at birth, asthma, chronic pneumonia and lung disease	Regular, unlabored, normal FVC and FEV1
Pulse/blood pressure	Regular, normal	Regular, normal
EKG	No information	Currently normal, but mother reported proband has had irregular heart rate
GI	Gastrostomy dependent, significant dysphagia. No organomegaly is appreciated. Occasional gastroesophageal reflux	None
Skin	Rash at 4–5 years; predominately on face but also on chest, back and bottom. Has erythematous base and there are papules and it did get both oozy and dry. Noted as atopic dermatitis. Periodic flair ups, but no record of rash after age 5.	Normal
Prior negative testing	Testing: metabolic panel, CBC, ferritin, serum carnitine, serum amino acids, free T4, TSH and IGF-1 testing did not reveal any endocrinologic abnormalities	Testing: Acid-alpha-glucosidase (GAA) activity (low but not deficient); Normal genetic sequencing: <i>GAA, DMD, FKRP, CAPN3, LMNA, CAV3, SGCA, SGCB, SGCD, SGCG, DYSF</i>
Other notes of interest	Normal brain MRI; abnormal EEG that was suggestive of seizures, but no seizures have been noted; bone age x-ray at 8yrs was normal; height and weight consistently under 3rd centile; abnormal sleep study	No obstructive sleep apnea; code 736 for club feet written on birth records but crossed off; complains of occasional joint pain

Table 1. Continued.

Family	F047		F045
Gender	Male (proband)	Male (father)	Male
Ethnicity			
Birth	G1, P0, ABO; vaginal, no known problems		G1, P0; 38 weeks; C-section; fraternal twins, IVF conception; APGAR 8, 9
Age of onset (retrospective)	Early infancy	Early infancy	Congenital
Age of first related clinical visit	7.5 month	~20 years	Birth
Age at time of study	6 years	43 years	9 month
Inheritance	Autosomal dominant		Autosomal recessive
Disease gene	RYR1		CACNA1S
Diagnosis based on genetic findings (OMIM#)	Central core disease (117000)		Novel Congenital myopathy with ophthalmoplegia
Contractures/joints	No fixed contractures, hyperflexibility and hypermobility	No fixed contractures	Legs flexed at birth, no fixed contractures, but some lower extremity flexion
Muscle biopsy histopathology and EM	No record of muscle biopsy	Muscle biopsy from many years ago yielded nonspecific findings	H&E, Gomori trichrome, NADH, ATPase (pH 4.3, 4.6, 9.4) revealed considerable myofiber size variation (5 to 20 μm) of both type I and type II myofibers, polygonal small and large fibers, and occasional internal nuclei. No myofiber degeneration, no fiber splitting, no endomysial or perifascicular connective tissue increase, no inflammation, no fiber type grouping, and no inclusions were detected. COX enzymatic activity staining demonstrated moderate architectural alterations in the form of coarse whorled fibers. Dysferlin, merosin, sarcoglycans (α, β, γ, δ), laminin, spectrin, emerin, dystrophins (I, II, III) and dystroglycans (α, β) IHC was normal. Normal lipid and glycogen content by Oil-red-O and PAS. EM revealed normal appearance, abundance, and distribution of mitochondria; no rods or cores.
CPK (international units)	<400	<400	<400 (multiple occasions)
Milestone delays	Delays in gross motor skills. roll over 6 months stand/walk 15+ months sit 18 months not regressing	Similar to son	n/a
Musculature changes	No obvious muscle wasting, no calf hypertrophy	Significant lower extremity calf hypertrophy	Normal without any obvious atrophy
Descriptions of muscle weakness	Somewhat myopathic facies, neck flexor strength decreased, proximal upper and lower extremities (4-)	Significant proximal upper and lower extremity weakness, myopathic facies	Severe generalized congenital weakness, hypotonia, pharyngeal phase dysphagia, oropharyngeal weakness, significant neck flexor and neck extensor weakness, has spontaneous movement of arms and legs.
Ophthalmoplegia, ptosis, nystagmus, facial diplegia	Mild facial diplegia, no ophthalmoplegia, no ptosis	Facial diplegia	Moderate facial diplegia, ophthalmoplegia developed by 3 months no ptosis
Fatigue	Exertional fatigue	Exertional fatigue	n/a
Deep knee bend test	Able to do 1 deep knee bend	Unable	n/a
Toe walk test	Able	No information	n/a
Heel walk test	Unable	No information	n/a
Gowers' test	Gowers'	No information	n/a
Gait	Abnormal gait with mild waddling quality	No information	n/a

(Continued)

Table 1. Continued.

Family	F047		F045
Choreiform/spasticity/tremor	None	None	None
Deep tendon reflexes	Normal to difficult to obtain	No information	Not obtainable
EMG	Normal	Normal	No information
Cognition and speech	Speech articulation problems	No information	Normal, alert, responds to parents voices
Dysmorphology	Elongated facies	Elongated facies	Mild micrognathia, high-arched palate, dolichocephalic and plagiocephalic
Respiration	regular, unlabored	No information	Somewhat labored and paradoxical
Pulse/blood pressure	Regular, normal	No information	Regular, normal
EKG	No information	No information	No information
GI	No information	No information	Gastrostomy dependent
Skin	No information	No information	Normal
Prior negative testing	No record of other prior related testing		Normal genetic testing: <i>SMN1</i> . <i>DMPK</i> repeat length. Chromosomal array and Prader–Willi methylation studies.
Other notes of interest		Undescended testicles	Uncontrolled fever 2–3 days, 103°F; normal brain MRI

Results

Collagen 6 myopathies

Case 1. F038 *COL6A3*

In Family 38, a male child of Canadian descent was born at term with club feet but no additional problems (Table 1). He walked somewhat late and since has had a consistently mild abnormal gait. At the age of 5–6 years, he underwent a period of weight loss and lipoatrophy, and at age 17 had a body mass index (BMI) in the first centile, and continued to have a malnourished appearance and difficulty gaining weight. From the age of 8 to age 17, he complained of significant weakness and fatigue. He displayed mild progressive proximal thoracic scoliosis and had significant but nonfixed bilateral lower extremity contractures of hamstrings, ankles, and feet with overlapping toes (Table 1). He had no history of skin rash. His respiration and cardiac function were normal. Testing revealed a consistent mild elevation of CPK. Electromyography (EMG) suggested chronic motor neuropathy. Abnormalities revealed by muscle biopsy histology included size variation, split fibers, internal nuclei, connective tissue proliferation, and endomysium proliferation (Fig. 1A). ATPase staining revealed moderate Type I and Type II grouping (Fig. 1B). NADH staining revealed moth-eaten fibers (Fig. 1C). The overall histological diagnosis was abnormal myofibrillar architecture with moderate fiber type grouping of unknown etiology. Genetic sequencing of 15 NMD genes was normal (Table 1). Over his 17 years of life, his diagnoses included Charcot–Marie–Tooth disease, myopathy, Pompe disease, MD, limb-girdle muscular dystrophy (LGMD), and spinal muscular

atrophy, but without genetic confirmation. There is no other known history of NMD in the family.

WES was performed on samples from the parents, proband, and sibling and revealed a rare homozygous missense variant in *COL6A3* (g.chr2:238253214T>C, c.7447A>G, p.Lys2483Glu, NM_004369.3) in the proband (Fig. 2 and Table 2). The variant was clinically confirmed by Sanger sequencing (data not shown). Each parent and the unaffected female sibling carried this variant in the heterozygous state (Figs. 2, 3A). The CADD score for this variant is 22.5. This variant is present in The Single Nucleotide Polymorphism Database (dbSNP) (rs139260335) (Sherry et al. 2001), and ExAC variant databases, but never in the homozygous state. This p.Lys2483Glu variant has been previously identified as pathogenic by Brinas et al. (2010) in a compound heterozygous state, but this is the first homozygous case to our knowledge.

Case 2 – F049 *COL6A3*

In Family 49, a male child was born as the product of a 24-year-old, gravida 2 para 1 mother. There were no known complications or health issues at birth. He began walking somewhat late at 14–15 months of age and has always had some gait difficulties (Table 1). He was evaluated at the age of 12 years and complains of fatigue and has been unable for several years to keep up with his peers physically. He experienced recent unexplained significant weight loss of 5 kg between the age of 11 and 12. He has no history of rashes or skin abnormalities. He was suspected of having respiratory insufficiency, but had forced expiratory volume 1 (FEV1) and forced volume vital capacity (FVC) of 100

Table 2. Novel myopathy pathogenic variants.

Family	F038	F049	F041	F043	F047	F045
Gene symbol (HGNC)	COL6A3	COL6A3	COL6A6	COL6A3	RYR1	CACNA1S
OMIM (gene)	120250	120250	-	120250	180901	114208
Cytogenetic locus (HGNC)	2q37	2q37	3q22.1	2q37	19q13.1	1q32
Variant state	Homozygous	Compound heterozygous	Compound heterozygous, possibly digenic with COL6A3	Heterozygous	Heterozygous	Compound heterozygous
Genomic position (GRCh37)	2:238253214	2:238253214	3:130279213	2:238253214	19:39075714	1:20102509
Variant (REF-ALT)	T>C	AC>A	T>A	T>C	C>CACCTTCTTCTTCTTCGTCACTC	C>A
Transcript ID (RefSeq)	NM_004369.3	NM_004369.3	NM_001102608.1	NM_004369.3	NM_000540.2	NM_000069.2
cDNA position	c.7447A>G	c.5480delG	c.5T>A	c.7447A>G	c.14778_14779insACCTTCTTCTTCGTCACTC	c.4947delA
dbSNP ID	rs139260335	rs139260335	-	rs139260335	-	-
Protein	Col6b3	Col6b3	Col6b6	Col6a3	RYR-1	Cav1.1
Protein effect	Missense	Frameshift	Missense	Missense	Insertion	Missense
Amino acid change	p.Lys2483Glu	p.Gly1827Valfs*1	p.Met2Lys	p.Lys2483Glu	p.Ile4926ins7 (TFFFRV)	p.Gln1265His
EXAC count (HOM/HET/Total ²)	0/68/122944	0/1/122894	0/0/-121792	0/68/122944	0/0/-120208	0/1/122872
Previously reported NMD gene?	Yes	Yes	No	Yes	Yes	Yes ¹
Present in normal databases?	EXAC, ESP, dbSNP	EXAC, ESP, dbSNP	None	EXAC, ESP, dbSNP	None	EXAC
Previously reported pathogenic variant?	Yes ³	No	No	Yes ³	No	No
CADD score (PHRED) (2/2015)	22.5	35	27.9	22.5	21.7	19.98

¹Mutations in CACNA1S are known to cause malignant hyperthermia susceptibility (MHS), and hypokalemic periodic paralysis type 1 (HOKPPI) but not myopathy described in this case.

²When variants were not present in the EXAC database, the allele count at the nearest variant in the database was used to approximate allele coverage at the locus.

³The p.Lys2483Glu pathogenic variant has been previously reported in a compound heterozygous case (Brinas et al. 2010).

and 105% of predicted. His cognition was normal. In-depth cardiac evaluation was normal. He demonstrated contractures of his hamstrings and ankles and previously wore orthotics to mitigate further ankle contractures. He had no upper extremity contractures but had some protuberance of bilateral scapula. Examination of his strength on multiple occasions by multiple physicians revealed 3–4/5 weakness in all limb and girdle muscles (Table 1). He had easily obtainable reflexes, but nerve conduction studies at the age of 9 identified fibrillations and sharp waves in the anterior tibialis. Extensive muscle biopsy histology revealed marked fiber size variation, with large hypertrophic and tiny multinucleated atrophic fibers with a majority showing central nuclei and occasional fiber splitting. Scattered regenerating and degenerating fibers were detected. Both Hematoxylin and Eosin (H&E) and trichrome stains revealed markedly increased connective and adipose tissue (Table 1). Of particular interest was the presence of Col6 staining of the endomysial connective tissue and sarcolemmal staining. However, the specific Col6 proteins detected were not reported. NADH reveals slight mottling of fibers with paler centers. Electron microscopy (EM) demonstrated some focal Z-band streaming involving only one sarcomere length. Serial CPK evaluations revealed mildly elevated values in the 500–700 range. His parents and female half sibling are unaffected and no other family history of similar disease was present (Fig. 3B).

WES revealed the same *COL6A3* pathogenic variant (g.chr2:238253214T>C, c.7447A>G, p.Lys2483Glu) identi-

fied in the first case, but only in the heterozygous state in case 2 (Figs. 3B, 4A, and Table 2). A second *COL6A3* frameshift variant (g.chr2:238275349AC>A, c.5480delG, p.Gly1827Valfs*17, NM_004369.3) was also identified in the proband. This variant has been reported in a single allele in ExAC. It is predicted to result in truncation of the protein, and likely does not result in translation of protein due to nonsense-mediated decay (NSMD) (Baker and Parker 2004). Variants were clinically confirmed by Sanger sequencing (Fig. 4A and B). The *COL6A3* p.Lys2483Glu pathogenic variant was inherited from the father and the p.Gly1827Valfs*17 pathogenic variant inherited from the mother (Fig. 3B). Our case is very similar to the case reported by Brinas et al. (2010) which had the p.Lys2483-Glu variant with a frameshift pathogenic variant.

Case 3. F041 *COL6A6* and *COL6A3*

In a third family, an infant female child was discovered during an ultrasound to have arthrogryposis. She was born prematurely at 35 weeks by emergency C-section. At birth, her APGAR score was 1. She was not breathing and was resuscitated. She presented further with micrognathia, dysphagia, dysarthria, and generalized hypotonia (Table 1). A feeding tube was implanted at 6 weeks of age. She has had significant milestone delays. She began to hold her head up, sit, smile, laugh, and roll over by ~6 months. She began to reach and grab for things at ~9 months. She began to stand at 2 years and began to

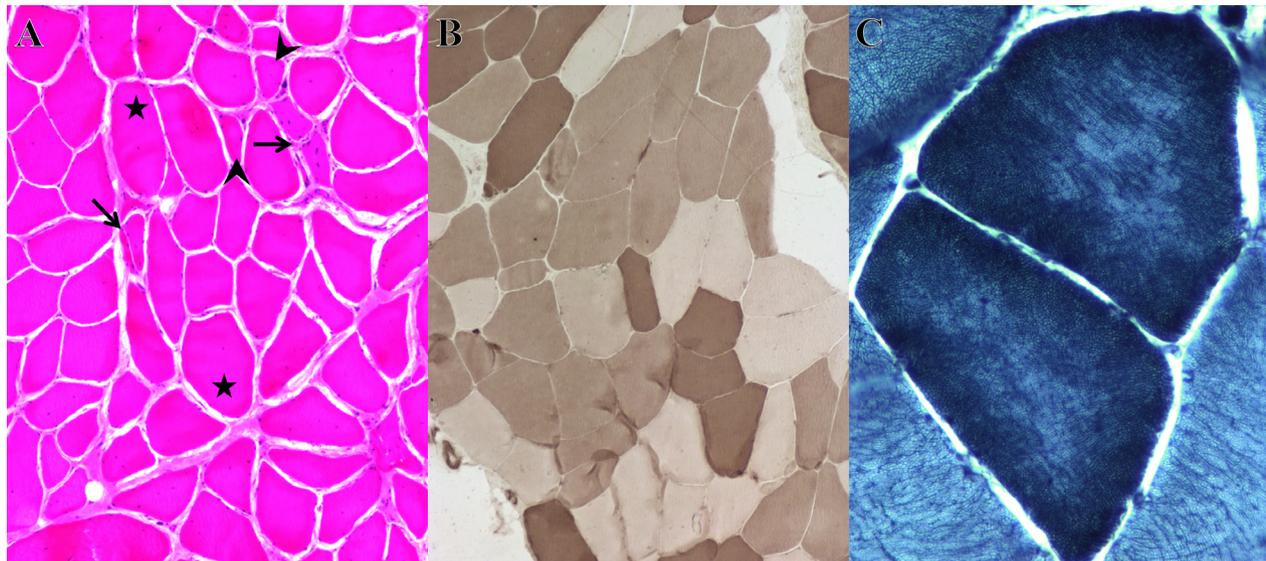


Figure 1. Histopathology images of frozen muscle biopsy cross sections from the affected male child in F038 carrying a homozygous p.Lys2483Glu *COL6A3* pathogenic variant. (A) H&E stain reveals atrophic (arrowheads), hypertrophic (stars), and split fibers (arrows). Also present are internal nuclei and connective tissue proliferation. Magnification = 100 \times . (B) ATPase reaction stain at pH 4.6 demonstrating that both fiber types are affected by hypertrophy and atrophy as well as mild fiber type grouping. Magnification = 100 \times . (C) NADH oxidative enzyme reaction stain demonstrating myofibrillar architectural disarray (moth-eaten fibers). Magnification = 400 \times .

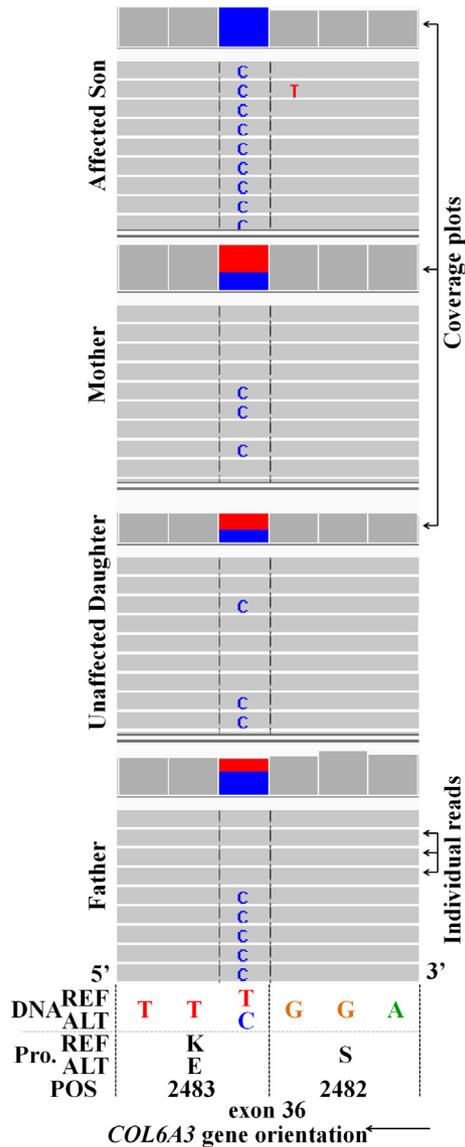


Figure 2. Integrated genomics viewer (IGV) screenshot of F038 WES results of the *COL6A3* c.7447A>G missense p.Lys2483Glu pathogenic variant. The affected son is homozygous for the T>C variant and all other family members represented are heterozygous. Note the DNA reference shows the complementary DNA sequence relative to the gene orientation. DNA reference (REF) based on hgGRCh37. Col6a3 transcript for protein position (Pro. POS) = NM_004369.

crawl at 3 years of age. At the age of 5 years, she began walking. She has very limited speech. She has consistent muscle weakness of all muscle groups that does not appear to be improving or progressing. At the time of this study, the child was 9 years of age. She has contractures of the fingers, wrist, elbow, shoulders, ankles, knees, and hips. Contractures present from birth have improved somewhat over the years (Table 1).

This family, including the proband, her parents, and her maternal grandmother were enrolled in our study. WES revealed the presence of the same *COL6A3* pathogenic variant (g.chr2:238253214T>C, c.7447A>G, p.Lys2483Glu) (Brinas *et al.* 2010) but only in a heterozygous state in the proband (inherited from her father) with no second pathogenic variant in *COL6A3* detected (Fig. 3C). Interestingly, we identified compound heterozygous variants in *COL6A6* in the proband, including a heterozygous missense g.chr3:130279213T>A, c.5T>A, p.Met2Lys (NM_001102608.1) variant inherited from her father and a heterozygous missense g.chr3:130361834 T>C, c.5194T>C, p.Cys1732Arg (NM_001102608.1) variant from her mother (Fig. 3C and Table 2). All three *COL6* variants in the proband were clinically confirmed by Sanger sequencing (Fig. 4C and D). The maternal grandmother did not carry any of these variants (Fig. 3C). The *COL6A6* variants have not been identified in dbSNP, ESP or ExAC, and received CADD scores of 22.6 (p.Met2Lys) and 27.9 (p.Cys1732Arg). While CADD scores, which take into account many predictors of pathogenicity, suggest these pathogenic variants are damaging, not all predictors suggest pathogenicity. The Residual Variation Intolerance Score (RVIS) of -0.11 (ALL 0.01% category) for the *COL6A6* gene as a whole compared to all other genes, is only very slightly intolerant of functional variation (Petrovski *et al.* 2013). Polyphen2 (Adzhubei *et al.* 2010) ranks both *COL6A6* variants as probably damaging with scores of 0.967 and 1.00 for the p.Met2Lys and p.Cys1732Arg variants, respectively. While we believe that the *COL6A6* variants are likely pathogenic, functional studies are warranted and our interpretation must be considered carefully until further *COL6A6* pathogenic variants are reported.

Emery–Dreifuss muscular dystrophy

Case 4. EMD F043

We evaluated a fourth case with similar clinical characteristics to the first two *COL6A3* cases. The male child was born at full term with mild complications with jaundice and wrapping of the umbilical cord around his neck and respiratory distress (Table 1). He began walking at 14 months and at ~4 years old was a toe walker. He was seen at age 6 for abnormal gait, slowly progressing extremity weakness in his feet and ankles, fatigue on excursion, and equinus foot deformity. Further evaluation at 11 years of age noted bilateral winged scapulae, rather significant progressive contractures at his elbows, moderate hamstring contractures, and moderate flexor contractures of his ankles, but no scoliosis (Table 1). He had a somewhat unusual gait

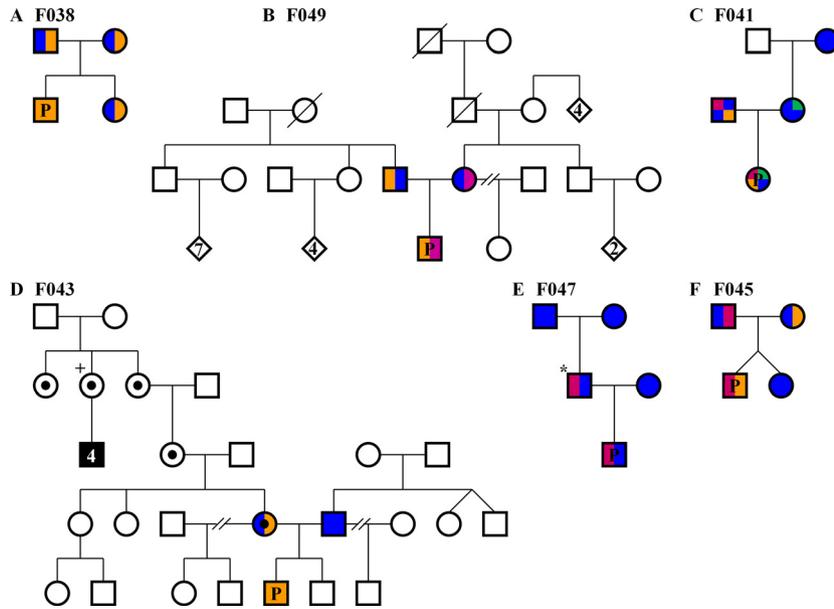


Figure 3. Pedigrees of families. Individuals with color filled symbols underwent exome sequencing. P, proband described in this study. ■ Represents wild-type alleles for the indicated gene in all pedigrees. (A) Family 38 pedigree. ■ *COL6A3* recessive c.7447A>G variant. (B) Family 49 pedigree. ■ *COL6A3* recessive c.7447A>G variant. ■ *COL6A3* recessive c.5480delG variant. (C) Family 41 pedigree. ■ *COL6A3* recessive c.7447A>G variant. ■ *COL6A6* recessive c.5T>A variant. ■ *COL6A6* recessive c.5194T>C variant. (D) Family 43 pedigree. ■ *EMD* recessive c.187+1G>T splice variant. + Required pacemaker and had four affected sons (black fill). ● Known obligate carrier. (E) Family 47 pedigree. ■ *RYR1* dominant c.14778_14779insACCTTCTTCTTCTCGTCATC variant. *De novo event. (F) Family 45 pedigree. ■ *CACNA1S* recessive c.4947delA variant. ■ *CACNA1S* recessive c.3795G>T variant.

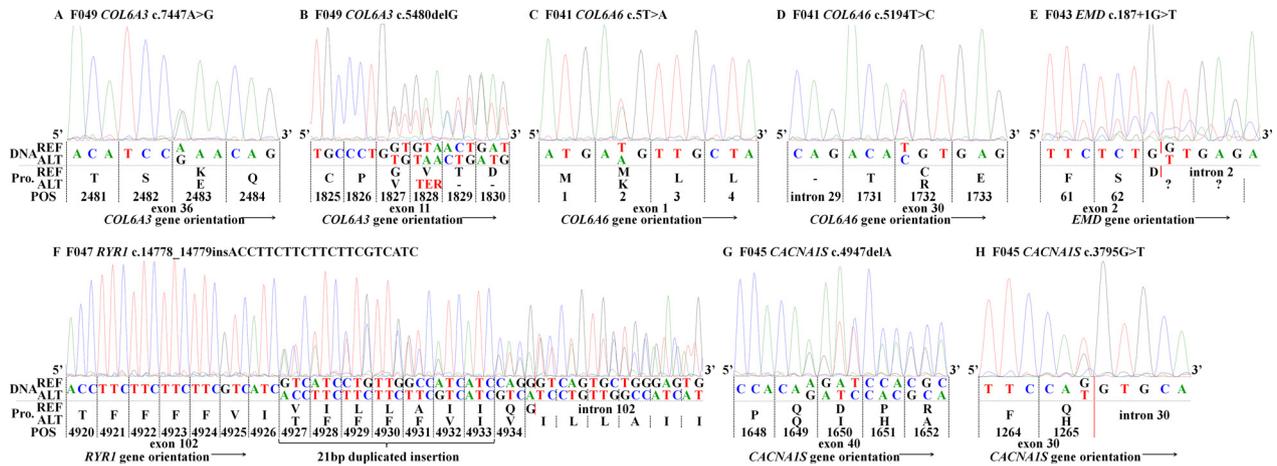


Figure 4. Sanger sequencing traces examples of each pathogenic variant reported from GeneDX clinical confirmation. DNA reference (REF) based on GRCh37. (A) F049 affected male child heterozygous *COL6A3* c.7447A>G recessive missense p.Lys2483Glu pathogenic variant. This trace is also similar to the results found in the F041 affected proband (*data not shown*). (B) F049 affected male child heterozygous *COL6A3* c.5480delG recessive frameshift p.Gly1827Valfs*1 pathogenic variant. (C) F041 affected female child heterozygous *COL6A6* c.5T>A recessive missense p.Met2Lys likely pathogenic variant. (D) F041 affected female child heterozygous *COL6A6* c.5194T>C recessive missense p.Cys1732Arg likely pathogenic variant. (E) F043 affected male child hemizygous *EMD* c.187+1G>T recessive pathogenic variant. (F) F047 affected male child heterozygous *RYR1* c.14778_14779insACCTTCTTCTTCTCGTCATC dominant duplicated insertion p.Ile4926ins7 (TFFFVI) pathogenic variant. (G) F045 affected male child heterozygous *CACNA1S* c.4947delA recessive frameshift p.Gln1649Glnfs*72 pathogenic variant. (H) F045 affected male child heterozygous *CACNA1S* c.3795G>T recessive missense p.Gln1265His pathogenic variant. Transcripts used for protein position (Pro. POS) for: Col6x3 = NM_004369; Col6x6 = NM_001102608; Emerin = NM_000117; RYR-1 = NM_000540; Cav1.1 = NM_000069. Red solid vertical bars represent splice sites. Dashed black vertical bars represent triplet codon reading frame.

with a mild waddling quality. He did not demonstrate any neck flexor/extensor or significant proximal weakness in his shoulder girdles. He did have clear distal weakness in foot dorsiflexors and decreased but obtainable deep tendon reflexes. Recent echocardiogram (EKG) and Holter monitoring revealed no structural heart disease, cardio myopathy, or arrhythmia. Muscle biopsy demonstrated mild myopathic changes without inflammation or significant fibrosis (Table 1). CPK values were slightly elevated and ranged from 500 to 800. EMG excluded a peripheral neuropathy and was suggestive of myopathy. His working diagnosis was an undetermined form of LGMD. Initially, no family history of related NMD was noted by the family.

The proband and his parents were enrolled in our study and WES revealed a novel pathogenic splice variant in *EMD* at g.chrX:153608155G>T, c.187+1G>T (NM_000117.2) for which the mother is heterozygous and the affected son is hemizygous (Fig. 3D and Table 2). This variant was clinically confirmed by Sanger sequencing (Fig. 4E). This c.187+1G>T splice variant has not been identified in dbSNP, or ExAC, but a c.187+1G>A splice variant has been reported to cause EDMD (Yates *et al.* 1999). After genetic diagnosis of the proband, several male members of the maternal extended family were identified that had previously been diagnosed and genetically confirmed with EDMD or cardiac conduction defects suggesting this variant is X-linked recessive. However, the sister of the maternal great grandmother required a pacemaker, possibly suggestive of a mild dominant effect or haploinsufficiency in heterozygous females (Fig. 3D).

Calcium channel myopathies

Case 5. *RYR1* F047

In F047, a male child was evaluated beginning at 7.5 months of age with delays in gross motor skills. He was not noted of having any problems at birth and went home from the hospital at a few days of life (Table 1). He had some milestone delays and was just beginning to roll over at ~6 month of age and began to walk at 15 months. He has had some speech articulation problems, but cognitive skills are described as normal. When examined at age 5 years of age he had somewhat elongated myopathic facies with mild facial diplegia. He fatigues somewhat more easily than his peers. He had no fixed contractures or obvious muscle wasting. He was able to do a single deep knee bend with difficulty. He rose from the floor with a significant Gowers' maneuver. His walking gait was abnormal, manifested by a mild waddling quality and his running gait was also abnormal. His

neck flexor strength is decreased. Examination of proximal strength in his upper and lower extremities revealed 4-/5 weakness (Table 1). His deep tendon reflexes were difficult to obtain. Approximately a year later at age 6 years, his evaluation was much the same, but it was noted that he was able, with difficulty, to walk on his toes, but was unable to walk on his heels. Also noted was a significant degree of hyperflexibility and hypermobility.

The father, age 43 at the time of this study, had a very similar early childhood history. He underwent an extensive neuromuscular evaluation approximately 20 years ago. The father's evaluation included EMG, normal plasma CPK, and a muscle biopsy which yielded nonspecific results. The father has a history of having generalized fatigue, and exertional weakness. He had no significant history of medical problems other than undescended testicles. At the time of our current study, he was reexamined and had clear evidence for significant proximal weakness in his upper and lower extremities, and could not do a single deep knee bend unaided (Table 1). He also had an elongated face with diplegia and myopathic looking facies. He exhibited significant lower extremity calf hypertrophy. The mother had no known neuromuscular disorder.

The boy, his parents, and paternal grandparents were enrolled in our study and WES revealed a novel small in-frame 21 bp duplicated insertion in *RYR1* beginning at g.chr19:39075714, c.14778_14779insACCTCTCTCTCTTCGTCATC, p.Ile4926ins7 (TFFFVVI) (NM_000540.2) (Fig. 5) in the proband and his affected father, but not present in either paternal grandparent indicating that the pathogenic variant is *de novo* in the father (Figs. 3E, 5). This variant was clinically confirmed by Sanger sequencing (Fig. 4F). This insertion has not been identified in dbSNP, or ExAC (Table 2). Pathogenic variants in *RYR1* are the most common cause of CCD (Wu *et al.* 2006), consistent with the phenotype of the father and affected son.

Case 6. F045 *CACNA1S*

The last case we describe was the product of a 38 week gestation of male–female twins conceived by IVF. The pair was born via C-section and the boy had APGAR scores of 8 and 9, but was described as being quite hypotonic, severely weak, not very vigorous, and never established the ability to significantly chew, suck, or swallow (Table 1). The sister had no noted problems. The boy was hospitalized for 2 months and underwent extensive neurodiagnostic evaluation. During this time, a gastrostomy tube was inserted. His height, weight, and basic vital signs have remained normal throughout his course. At his evaluation at 3 month of age, he was somewhat dolichocephalic and plagiocephalic, for which he wore a helmet. He had a high-arched palate with mild micrognathia. He

had clear pharyngeal phase dysphagia, oropharyngeal weakness, significant facial diplegia, but no apparent ptosis. He had no fixed contractures, but did have some flexion in his lower extremities (Table 1). He was alert and responded to his parents' voice and followed them visually, but he moved his head more than his eyes clearly demonstrating ophthalmoplegia. He had spontaneously movement of his hands and feet. Deep tendon reflexes were not obtainable. Muscle biopsy and extensive histology demonstrated moderate myofiber atrophy and hypertrophy of both fiber types with myofiber size variation ranging from 5 to 20 microns (Fig. 6A). Fibers were polygonal with no myofiber degeneration or splitting. Occasional internal nuclei were present. No fiber type grouping and no inflammation or inclusions were seen (Fig. 6B). Phosphofructokinase (F-6-P) staining revealed course myofibrillar architecture (Fig. 6C). Other histological stains did not reveal any further abnormalities (Table 1). High-resolution magnetic resonance imaging (MRI) did not reveal any structural abnormalities in the brain, cerebellum, and brainstem.

The boy, his parents, and his unaffected fraternal female twin were enrolled in our study. Analysis of variants identified by WES revealed novel pathogenic variants in *CACNAS1* in the family. The affected son received a pathogenic single bp deletion at g.chr1:201012509CT>C, c.4947delA, p.Gln1649Glnfs*72 (NM_000069.2) from his father and a missense g.chr1:201022587C>A c.3795G>T, p.Gln1265His (NM_000069.2) from his mother (Fig. 3E). These variants were clinically confirmed by Sanger sequencing (Fig. 4G and H). The missense p.Gln1265His variant is predicted to be damaging by FATHMM, Polyphen-2, mutationassessor (Reva et al. 2007, 2011), and

MutationTaster. No prediction algorithms used assigned this variant as benign. These *CACNAS1* variants have not been reported in dbSNP, or ESP, but are each present in a single allele in ExAC (Table 2). The twin sister of the affected boy was discordant for both *CACNAS1* variants (Fig. 3E). The clinical features of the disease in Case 6 are consistent with a severe congenital myopathy with ophthalmoplegia. *CACNAS1* pathogenic variants have been identified as the cause of hypokalemic periodic paralysis type 1 (HOKPP1) (Burge and Hanna 2012; Hanchard et al. 2013), but persistent weakness of the child is somewhat inconsistent with HOKPP1. While it has been suspected that *CACNAS1* variants could cause myopathies due to the physical and mechanical coupling between its gene product, the voltage-dependent L-type calcium channel subunit alpha-1S (Cav1.1), and the Ryanodine receptor Ca²⁺ release channel 1 (RYR-1) (Wu et al. 2006; Rebbeck et al. 2014), no reports prior to this have identified pathogenic variants in *CACNAS1* as the cause of congenital myopathy.

Discussion

Congenital and childhood myopathies and muscular dystrophies are a common class of neuromuscular disorders with overlapping phenotypes and heterogeneous genetic etiology. Here, we present the phenotype of 6 myopathy cases that underwent extensive, expensive, and invasive testing including muscle biopsies, EMG, MRI, EKG, and single gene and gene panel sequencing, often over many years, without successful genetic diagnosis prior to enrollment in our research study (Table 1). We identified novel pathogenic variants in five genes by WES (Table 2).

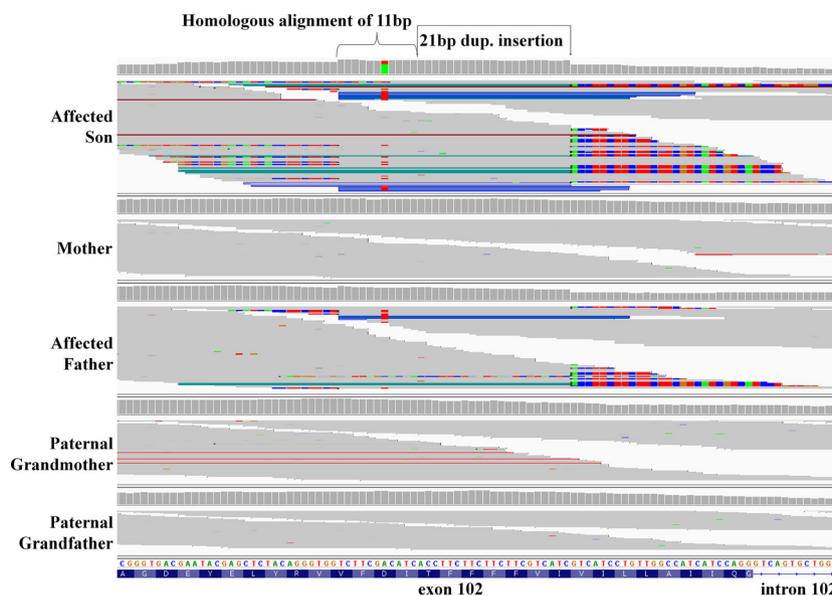


Figure 5. IGV screenshot of the F047 WES results demonstrating a novel 21 bp duplicated *RYR1* c.14778_14779insACCTTCTTCTTCGTCATC dominant in frame p.Ile4926ins7 (TFFFVI) pathogenic variant. Note the homologous alignment of 11 bp causing the insertion to appear to be 32 bp in length. The insertion is present in the affected son and father but not in the paternal grandparents demonstrating a *de novo* dominant event in the father. *RYR-1* transcript = NM_000540.

Collagen 6 myopathies (cases 1-3)

We identified a recessive novel pathogenic variant in *COL6A3* and likely pathogenic variants in *COL6A6*. The family of Col6 proteins are extracellular matrix (ECM) proteins that help maintain tissue integrity of many tissues including muscle, tendon, skin, cartilage, and intervertebral disks (Chu *et al.* 1988; Knupp and Squire 2001; Bushby *et al.* 2014). The main forms of Col6 expressed in the ECM are the Collagen 6 alpha 1 (Col6 α 1), 2 (Col6 α 2), and 3 (Col6 α 3) chains coded for by *COL6A1*, *COL6A2*, and *COL6A3*, respectively (Engvall *et al.* 1986; Bonaldo *et al.* 1998). Pathogenic variants in *COL6A1*, *COL6A2*, and *COL6A3* cause two main forms of myopathy; Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) (Bonnemann 2011). Recently, the *COL6A6* gene was discovered. It is most similar to *COL6A3* as it codes for multiple von Willebrand factor type A (VWFA) domains (Gara *et al.* 2008; Tagliavini *et al.* 2014). *COL6A6* is expressed in wide range of fetal and adult tissue including brain, heart, and muscle (Fitzgerald *et al.* 2008; Gara *et al.* 2011). Col6 α 3 and Collagen 6 alpha 6 (Col6 α 6) proteins are found in the endomysium and perimysium of skeletal muscle but only Col6 α 3 is found in the basement membrane (Sabatelli *et al.* 2011, 2012). Recent evidence demonstrates that Col6 α 6 is dramatically decreased in skeletal muscle and muscle cell cultures from patients with UCMD and BM independent of

clinical phenotype suggesting coregulation of these genes (Tagliavini *et al.* 2014). Col6 α 6 was increased in noncollagen myopathies suggesting a significant role in other myopathies as well (Tagliavini *et al.* 2014).

Characteristic UCMD presents with severe congenital muscle weakness with axial and proximal joint contractures and distal joint hypermobility. BM usually presents with slowly progressive axial and proximal muscle weakness with finger flexion contractures. Skin rashes often accompany BM and UCMD (Bonnemann 2011; Bushby *et al.* 2014). Many pathogenic variants in Collagen 6 genes (*COL6*) impair protein expression by disrupting splicing, glycine substitutions required for triple-helix formation, or secretion. However, some pathogenic variants in BM patients have no detectable effect on Col6 assembly and secretion but compromise protein function in the ECM of muscle. Pathogenic variants in *COL6* genes have been reported as dominant and recessive (Lampe *et al.* 2008; Butterfield *et al.* 2013). The three cases describe here all display recessive phenotype since no carriers have yet displayed a phenotype. Interestingly, all three patients have the pathogenic *COL6A3* p.Lys2483Glu variant first reported by Brinas *et al.* (2010). Case 1 is homozygous for this variant. Case 2 is compound heterozygous for the p.Lys2483Glu variant and a *COL6A3* p.Gly1827Valfs*1 frameshift variant that would likely result in truncation and loss of expression. The phenotype of case 1 and case 2 are remarkably similar and

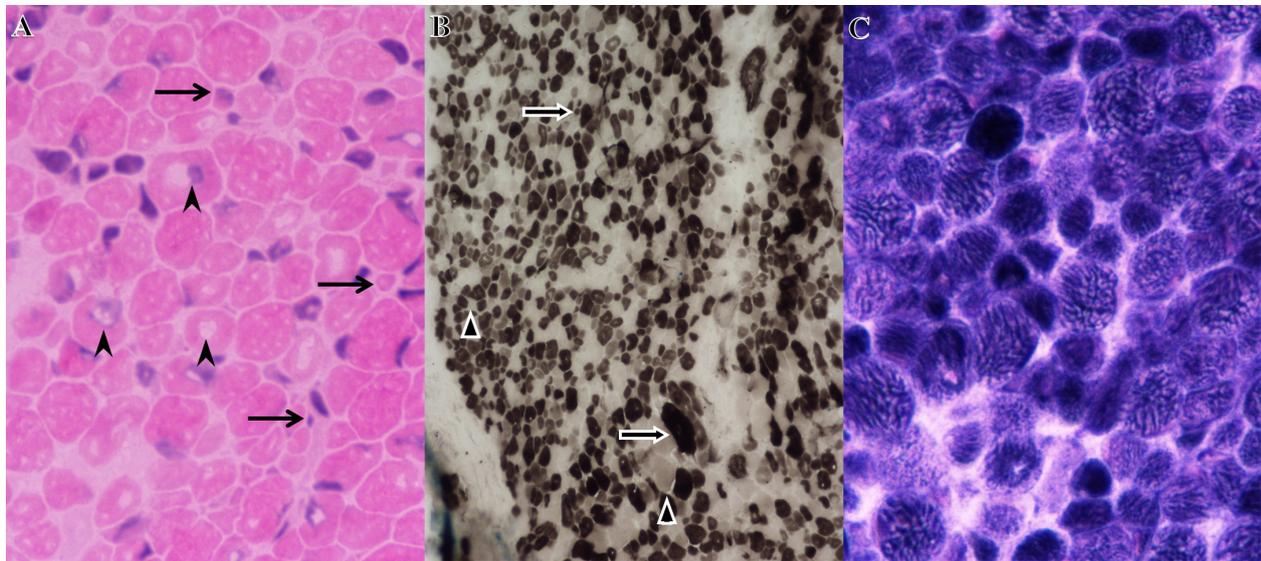


Figure 6. Histopathology images of frozen muscle biopsy cross sections from the affected male child in F045 carrying compound heterozygous p.Gln1649Glnfs*72 and p.Gln1265His *CACNA1S* pathogenic variants. (A) H&E stain demonstrating marked variability in myofiber size and fiber diameters ranging from 5 to 20 μ m. Arrows indicate tiny atrophic fibers. Central nuclei indicated by arrow points. Magnification = 600 \times . (B) ATPase reaction stain at pH 4.6 demonstrating that both fiber types are affected by hypertrophy and atrophy. Arrows indicate Type I (dark fibers) and arrowheads indicate Type II (light fibers). Magnification = 200 \times . (C) F-6-P stain demonstrating coarse myofibrillar architecture. Magnification = 600 \times .

indicate a myopathy more akin to the more mild BM form of disease. Their mild phenotype is very similar to that reported by Brinas et al. (2010) with the exception that our case demonstrated contractures. The F041 case had some additional features apart from the other two and is more suggestive of a more severe form of UCMD. The affected child carries novel compound heterozygous variants in *COL6A6* (p.Met2Lys and p.Cys1732Arg) in addition to being a heterozygous carrier of the *COL6A3* p.Lys2483Glu pathogenic variant. The *COL6A6* variants are likely pathogenic and we believe are the primary drivers of the phenotype, but the *COL6A3* variant likely contributes to the phenotype due to the overlap in expression and function. While it is clear there is a genetic component to her condition, we cannot rule out that complications at birth did not also contribute to her more severe phenotype.

It is clear that the *COL6A3* p.Gly1827Valfs*1 variant in F049 is pathogenic and is predicted to result in NSMD or protein truncation. The p.Lys2483Glu pathogenic variant in *COL6A3* causes a change in amino acid structure and opposite charge. It is located in the nonhelical region of the protein, in the VWFA 11 domain (Pan et al. 1998). Missense mutations in VWFA domains have been reported to cause BM (Pan et al. 1998). Changes in *COL6A3* expression or function of the p.Lys2483Glu variant at the endomysium and the perimysium is likely related to ECM proliferation identified in histology of F038 and F049 (Sabatelli et al. 2011; Bushby et al. 2014). The histology report for F049 indicates Col6 is present suggesting that the p.Lys2483Glu variant may not result in loss of expression or secretion, however, this must be considered cautiously as the pathology report does not indicate which Col6 proteins are detected. In the Brinas et al. (2010) case, Col6 was detected but altered in fibroblasts and not detected in muscle. In *COL6A6*, the p.Met2Lys missense pathogenic variant is located in the signal peptide of the protein and may disrupt its secretion. This variant may also disrupt translation of the *COL6A6* mRNA as mutations in stem loop structures near the start codon of other collagen genes abolish expression (Manojlovic and Stefanovic 2012). The p.Cys1732Arg pathogenic variant in *COL6A6* results in an addition of a charge to the amino acid and a change from hydrophobic to hydrophilic states. This variant is also located at the end of the triple-helical domain and just before the C-terminal VWFA domains (Pan et al. 1998). In general, Cys residues are highly conserved and are critical for formation of collagen oligomer and fibril disulfide bonds (Butterfield et al. 2013).

Of particular interest is the specific period of lipoatrophy and weight loss, and the inability to gain weight in cases 1 and 2, a characteristic of patients not typically

described in cases of UCMD or BM (Brinas et al. 2010; Bonnemann 2011; Bushby et al. 2014), but which may be a diagnostic feature of disease caused by these variants. Weight loss and fatigue may be due to nocturnal respiratory insufficiency or apnea common to BM cases with *COL6A3* pathogenic variants (Bonnemann 2011). While suggested in case 2, neither case 1 nor 2 demonstrated waking respiratory insufficiency upon testing. Case 2 suffered from intermittent sleeping problems, but a sleep study did not reveal any apnea. Alternatively, several papers demonstrate metabolic changes in UCMD and BM including mitochondrial deficits in mouse models, cell models from UCHM and BM patients, and in patient muscle biopsies (Tagliavini et al. 2013; De Palma et al. 2014; Sorato et al. 2014). While muscle biopsy histology and EM did not reveal defects in morphology or number of mitochondria, respiratory chain deficits may explain the weight loss and fatigue seen in these patients.

Together, these three cases provide substantial evidence of the identification of pathogenic variants in *COL6A3* and likely pathogenic variants in *COL6A6*. While the *COL6A3* p.Lys2483Glu pathogenic variant is rare, it has a relatively high heterozygous frequency (Table 2) in the population. Therefore, it is critical to report this pathogenic variant and it is very likely that many more genetically undiagnosed cases of BM will be found to have this variant.

Emery–Dreifuss muscular dystrophy (case 4)

In addition to novel Col6 myopathies, we describe a novel c.187+1G>T pathogenic *EMD* splice variant in a single case of EDMD. EDMD usually manifests in childhood with slowly progressive weakness and limb muscle wasting. Contractures of the elbows, Achilles tendons, and postcervical muscles, are early and characteristic features. Cardiac conduction defects including arrhythmias and risk of sudden death are consistent features as well (Emery 1989; Bonne et al. 1993). The phenotype of the affected child in F043 is consistent with EDMD. The pathogenic variant we identified destroys the required canonical GT nucleotides of the 3' splice site of exon 2 of the *EMD* gene. While pathogenic variants have been identified throughout the *EMD* gene, exon 2 has been reported as a mutational hotspot (Brown et al. 2011). This c.187+1G>T splice variant is also at the exact position of a previously reported c.187+1G>A pathogenic variant in EDMD (Deymeer et al. 1993; Yates et al. 1993, 1999). The G>A and G>T splice variants undoubtedly have very similar if not identical effects on *EMD* gene splicing and expression. The phenotype of F043 is consistent with phenotypes reported for families with the G>A splice variant (Deymeer et al. 1993; Yates et al. 1993,

1999). The identification of a second variant at the same genomic locus in individuals affected with EDMD confirms both variants as pathogenic.

F043 also demonstrates the importance of obtaining a genetic diagnosis for myopathy patients. In EDMD, cardiac dysfunction is a primary cause of premature mortality if left unmonitored and untreated. As described here, children with similar phenotypes (BM and EDMD) have very different requirements when it comes to cardiac assessment and treatment.

***RYR1* and *CACNA1S* calcium channel myopathies (cases 5-6)**

Finally, we describe two calcium channel myopathy cases. The first is a dominant case caused by a novel p.Ile4926ins7 pathogenic variant in the well-known CCD gene *RYR1*. Second, we describe a novel severe form of congenital myopathy with ophthalmoplegia caused by compound heterozygous pathogenic variants in *CACNA1S* (p.Gln1649Glnfs*72 and p.Gln1265His). Several calcium channelopathies are associated with pathogenic variants in *RYR1* and *CACNA1S*. Malignant Hyperthermia Susceptibility (MHS) is caused by pathogenic variants in *RYR1* or *CACNA1S* (Monnier *et al.* 1997; Kim *et al.* 2013). Pathogenic variants in *CACNA1S* have been identified as a cause of HOKPP1, but *RYR1* have not (Burge and Hanna 2012; Hanchard *et al.* 2013). Pathogenic variants in *RYR1* are the most common causes of CCD, while *CACNA1S* have not been identified as a cause of CCD (Wu *et al.* 2006). CCD is most often characterized clinically by a stable or slowly progressive course of congenital hypotonia but can be more severe (Bharucha-Goebel *et al.* 2013). Most *RYR1* pathogenic variants usually result in a dominant phenotype consistent with the dominant heterozygous p.Ile4926ins7 pathogenic variant identified by WES in F047. Of special interest in this case is that the pathogenic variant in the affected father is *de novo*, which he then passed on to his affected son.

RYR1 and *CACNA1S* genes code for integral components of excitation-contraction coupling (EC) in skeletal muscle. EC depends on a physical interaction between the skeletal forms of the dihydropyridine receptor L-type Ca²⁺ channel (DHPR) and RYR-1 (Paolini *et al.* 2004; Polster *et al.* 2012). The *CACNA1S* gene codes for Cav1.1, the main subunit of the DHPR channel (Rebeck *et al.* 2014). When a voltage neurostimulus is received, the DHPR channel changes conformation and physically causes RYR-1 to open and release sarcoplasmic reticulum calcium (Rebeck *et al.* 2014).

The F047 p.Ile4926ins7 in *RYR1* is located in the final hydrophobic transmembrane domain of RYR-1 which

has been designated as a pathogenic variant hotspot (MacLennan and Zvaritch 2011). This insertion is in-frame, likely expressed, and is predicted to result in addition of seven amino acids in the final transmembrane helix nearest the Ca²⁺ pore formed by RYR-1. These extra amino acids undoubtedly alter RYR-1 function and folding. Since this variant shows dominant inheritance, it likely has a dominant negative effect by poisoning RYR-1 tetramer formation and function (MacLennan and Zvaritch 2011).

In F045, the severe congenital myopathy with ophthalmoplegia strongly suggested pathogenic variants in *RYR1*, but no pathogenic variants in *RYR1* were identified. Instead, we identified novel pathogenic frameshift (p.Gln1649Glnfs*72) and missense (p.Gln1265His) variants in *CACNA1S*. The frameshift variant likely abolishes translation of the mRNA. If the resulting mRNA is translated to protein, it is predicted to result in premature truncation later in the protein. Cav1.1 has four transmembrane domains each with six transmembrane helices that form the Ca²⁺ channel. The Cav1.1 p.Gln1265His pathogenic variant is located in its fourth repeat domain in the short cytoplasmic loop linking the transmembrane helix S4 and the positively charged transmembrane helix S5. This cytoplasmic loop may be involved in interactions with RYR-1 or other components of the DHPR (Rebeck *et al.* 2014). The change in amino acid structure may also interfere with DHPR calcium flux. Due to the phenotypic overlap with *RYR1* pathogenic variants in F045, we hypothesize that the p.Gln1265His variant disrupts DHPR and RYR-1 coupling. While pathogenic variants in *CACNA1S* have been demonstrated as the cause of MHS and HOKPP1, to our knowledge, this is the first reported case of severe congenital myopathy with ophthalmoplegia resulting from pathogenic variants in *CACNA1S*.

Reporting these cases not only raises awareness to extensive clinical overlap between similar cases with different genetic etiology but most importantly highlights the utility of WES and WGS in providing genetic diagnosis in clinically enigmatic cases.

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Conflict of Interest

None declared.

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Supporting Information

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

Figure S1. Exome target coverage statistics. The percent of bases of the targeted exome that had at least 20 high quality reads covering each base.