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A Mutation in *MTM1* Causes X-Linked Myotubular Myopathy in Boykin Spaniels.

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Abstract

The purpose of this study was to report the findings of clinical and genetic evaluation of a 3-month old male Boykin spaniel (the proband) that presented with progressive weakness. The puppy underwent a physical and neurological examination, serum biochemistry and complete blood cell count, electrophysiological testing, muscle biopsy and whole genome sequencing. Clinical evaluation revealed generalized neuromuscular weakness with tetraparesis and difficulty holding the head up and a dropped jaw. There was diffuse spontaneous activity on electromyography, most severe in the cervical musculature. Nerve conduction studies were normal, the findings were interpreted as consistent with a myopathy. Skeletal muscle was grossly abnormal on biopsy and there were necklace fibers and abnormal triad structure localization on histopathology, consistent with myotubular myopathy. Whole genome sequencing revealed a premature stop codon in exon 13 of *MTM1* (ChrX: 118903496 C > T, c.1467C>T, p.Arg512X). The puppy was humanely euthanized at 5 months of age. The puppy's dam was heterozygous for the variant, and 3 male puppies from a subsequent litter all of which died by 2 weeks of age were hemizygous for the variant. This naturally occurring mutation in Boykin spaniels causes a severe form of X-linked myotubular myopathy, comparable to the human counterpart.

Keywords

Canine; Centronuclear myopathy; Myotubularin myopathy

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1. Introduction

Centronuclear myopathies are rare but devastating causes of congenital myopathy grouped together based on their characteristic histopathological findings [1]. Within this group of myopathies, X-linked myotubular myopathy (XLMTM, OMIM 310400) caused by mutations within the myotubularin (*MTM1*) gene form a particularly severe subset [2]. In the classic form of XLMTM, affected infant boys show signs of facial, axial and proximal muscular weakness, hypotonia and areflexia leading to respiratory insufficiency and death soon after birth. If affected individuals survive the immediate post-natal period, feeding and ventilatory support are necessary and 25% of affected individuals die within the first year of life [3,4]. Less severe forms have also been described in males [5]. Women carrying the mutation were previously thought to be clinically normal, but more recent work suggests that they too can manifest a wide range of severities of clinical signs, in which asymmetry and facial involvement are frequent [6].

The *MTM1* gene is expressed ubiquitously and functions as a phosphoinositide phosphatase. While the full functional mechanisms are still being investigated, myotubularin plays an important role in membrane trafficking and in integrin-mediated maintenance of myofiber organization [7]. Defects in myotubularin result in disorganization of the T-tubule system, excitation-contraction coupling defects and abnormal calcium release [8–10]. Over 300 mutations in *MTM1* have been described in people with XLMTM (https://databases.lovd.nl/shared/variants/MTM1/). These are evenly distributed over the gene, mostly involve mRNA degradation, and result in total loss of myotubularin[11]. Models of this disease include a mouse knockout [12,13] and a zebra fish morpholino [9]. Naturally occurring *MTM1* mutations have been recognized in the Labrador retriever on exon 8 [14,15] and the Rottweiler on exon 11[16] both causing progressive tetraparesis and death. In this report we describe a new spontaneously occurring *MTM1* mutation in Boykin Spaniels with significant clinical abnormalities consistent with XLMTM and compare the clinical and histopathological findings with those of other dog breeds and humans.

2. Methods

2.1 Animals.

A 3-month-old male Boykin Spaniel puppy was presented to the North Carolina State Veterinary Hospital for chronic and progressive weakness and underwent a full diagnostic work up. Evaluations included obtaining a full medical history of the affected dog, littermates, sire and dam, physical and neurological examinations, complete blood cell count, serum biochemistry panel, urinalysis and thoracic radiographs of the affected dog. Remaining EDTA blood was used for genomic DNA extraction using the Gentra Purgene DNA Extraction Kit (Qiagen). An electrophysiological work up (electromyography (EMG), motor nerve conduction studies, repetitive nerve stimulation, an F-wave study and cord dorsum potential studies) was completed under general anesthesia. Anesthesia involved premedication with methadone (0.5mg/kg IM) and glycopyrrolate (0.01mg/kg IM) and induction of anesthesia with midazolam (0.28mg/kg IV) and propofol (2mg/kg IV). Following intubation, anesthesia was maintained with an inhaled sevoflurane and oxygen

mixture with a CRI of fentanyl. All electrophysiological studies were performed on the right side using routine methods[17] with a Nicolet VikingQuest, Natus Neurology Incorporated, Middleton, WI, USA. Following electrophysiology, biopsies were collected from the left triceps brachii, vastus lateralis and cranial tibial muscles. The dog was then recovered from anesthesia and *toxoplasma gondii* and *neospora caninum* serology was submitted. The dog returned home following the evaluation and progress was documented through telephone conversations with the owners.

Saliva or whole blood samples were collected from an additional 17 Boykin Spaniels including both parents, 10 siblings, 1 half sibling and 4 unrelated dogs, and DNA was extracted via standard protocols (Performagene saliva kit, QIAmp DNA Blood Kit).

2.2 Histopathology, Immunohistochemistry and Western Blotting.

Unfixed muscle biopsies were either wrapped in a saline dampened gauze sponge and chilled or immersion fixed in 10% neutral buffered formalin. Biopsies were shipped by an overnight service under refrigeration to the Comparative Neuromuscular Laboratory at the University of California, San Diego, immediately flash frozen in isopentane pre-cooled in liquid nitrogen, and stored frozen at −80°C until further processed. Cryosections were stained or reacted with a standard panel of histochemical stains and reactions [18]. Fixed biopsies were paraffin embedded and processed by standard procedures. Additional cryosections (8 μm) were cut and stained by indirect immunofluorescence as previously described [16,19]. Sections were incubated with a rabbit polyclonal antibody against the T-tubule marker dihydropyridine receptor (DHPRα1, (1:100 dilution, AbCam, Cambridge,UK) and a mouse monoclonal antibody against the sarcoplasmic reticulum (SR) marker RyR1 (1:100 dilution, Abcam, Cambridge, UK).

Western blotting was performed by standard methods using extracts from the vastus lateralis muscle of the affected dog and archived cryopreserved control muscle. Protein bands were separated using NuPage Bis-Tris 4-12% gradient gels (Invitrogen). Primary antibodies included an antibody against myotubularin (F-1, 1:500, Santa Cruz Biotechnology SC-377309) and an antibody against β-actin (1:2000, Sigma A2066) as a loading control. Secondary antibodies included peroxidase conjugated goat anti-mouse IgG (1:20,000, Jackson ImmunoResearchLab, 115-035-062) and peroxidase conjugated goat anti-rabbit IgG (1:20,000, Thermo Scientific, 31460). Protein bands were detected using Super Signal West Dura Extended Duration Substrate (Thermo Scientific).

2.3 DNA Sequencing.

Approximately 3 μg of genomic DNA from the proband was submitted for library preparation and whole genome sequencing at Genewiz LLC Next-Generation Sequencing Laboratory, using a 150 bp paired-end read configuration in a single lane of an Illumina HiSeq 4000 high-throughput sequencing system. These reads have been made publicly available at NCBI's Short Read Archive at http://www.ncbi.nlm.nih.gov/bioproject/556278. Variant calling from WGS data was performed using a standardized bioinformatics pipeline for all samples as described previously.[20] Briefly, sequence reads were trimmed using Trimmomatic 0.32[21] to a minimum phred-scaled base quality score of 30 at the start and end

of each read with a minimum read length of 70 bp, and aligned to the canFam3 reference sequence [22]using BWA 0.7.13[23]. Aligned reads were prepared for analysis using Picard Tools 2.5 (http://broadinstitute.github.io/picard) and GATK 3.7[24] following best practices for base quality score recalibration and indel realignment (Broad Institute, Cambridge, MA) [25,26]. Variant calls were made using GATK's HaplotyeCaller walker, and variant quality score recalibration (VQSR) was performed using sites from dbSNP 146 and the Illumina 174K CanineHD BeadChip as training resources. We applied a VQSR tranche sensitivity cutoff of 99.9% to SNPs and 99% to indels for use in downstream analyses; genotype calls with a phred-scaled quality score < 20 were flagged but not removed from the variant callset.

Heterozygous or homozygous variants present in the proband were filtered against a population of 187 non-Boykin Spaniel dogs of 31 different breeds whose entire genomes were sequenced as part of ongoing work in our laboratory. These breeds included: 22 Boxers, 20 Standard Poodles, 19 Great Danes, 13 Yorkshire Terriers, 11 Cavalier King Charles Spaniels, 10 Dachshunds, 10 Miniature Poodles, and 82 dogs of 25 additional breeds. Whole genome sequences from these dogs were processed using a similar bioinformatics pipeline as described above. None of these dogs were known to have any type of myopathy, junctionopathy, or lower motor neuron disease.

Variants that passed our filtering step and were unique to the proband were annotated using Variant Effect Predictor 91[27], and evaluated based on the severity of the predicted effect. Variants we considered most impactful included frameshifts, in-frame insertions and deletions, premature-start, stop-gained, missense, and splice region changes.

Primers were designed for variants identified as candidates using Primer3 [28,29] and the variants were confirmed using Sanger sequencing. Specifically, polymerase chain reaction was used to amplify a 201 bp sequence surrounding a deleterious variant in the *MTM1* gene using the following primers: F-5'-CGACAATGTTGCATTACTTGGG-3' and R-5'-TCCCGAGCAGATTCACAGTT-3'. PCR was performed on a BioRad S1000 thermal cycler using DreamTaq PCR Master Mix (Thermo-Fisher Scientific) standard protocol, and products were sent to Genewiz LLC for Sanger sequencing. This variant was investigated for conservation across species, and effect on protein structure by RaptorX.[30] All samples from Boykin spaniels were tested for the *MTM1* variant using the PCR protocol described above.

3. Results

The proband was 3 months of age at time of work up. The affected puppy was from a litter of 11 puppies, 3 females, all healthy and 8 males, 5 of which were small at birth and died within 2 weeks. At 3 weeks of age the proband stopped nursing and had to be fed from a bottle. Using this approach, the puppy could suckle well and continued to grow. At 5 – 6 weeks of age the owners noted an uncoordinated gait, difficulty rising after sleeping and difficulty descending stairs. Motor skills were delayed compared to those of the surviving littermates. The owners also noted that the jaw frequently hung open and the puppy was always thirsty. The appetite was excellent although support was needed to eat and drink.

At around 8 weeks of age the owners found the puppy asleep and they could not rouse him. They took him to an emergency veterinarian where recovery was spontaneous. Treatment was initiated for intestinal worms with fenbendazole (50 mg/kg for 3 doses) for possible protozoal disease with clindamycin (11 mg/kg twice daily) and empirically with prednisone (2mg/kg/day). A routine CBC and serum biochemistry panel performed 2 days later were unremarkable, but a bile acid tolerance test showed elevated postprandial levels (4.7 umol/L pre and 33.7 umol/L post; normal range: 0-50umol/L post) suggestive of a portosystemic shunt. This diagnosis was excluded at 2 months of age following liver ultrasonography, ammonia concentrations, repeat bile acid tolerance test. A fluoroscopic swallowing study demonstrated oropharyngeal dysphagia, consistent with neuromuscular disease. Given the progressive weakness, the puppy was presented to the NC State Veterinary neurology service for further evaluation.

At time of neurological assessment, the dog was mentally appropriate with normal cranial nerve examination except for a dropped jaw. The puppy was ambulatory tetraparetic with generalized weakness, a dropped head when walking, and he rapidly tired and became recumbent. (Supplementary Video 1) With appropriate weight support, proprioceptive placing and hopping were normal. Withdrawal reflexes were markedly reduced in all 4 limbs and the patellar reflexes were barely present. Diffuse muscle atrophy was present. Pain was not detected on palpation. The neuroanatomic diagnosis was diffuse neuromuscular disease.

Routine blood work showed an elevated ALT (528 IU/L; normal range: 12 - 54 IU/L) and low creatinine (0.3 mg/dL; normal range: 0.7 – 1.5mg/dL) but was otherwise unremarkable (other changes present were consistent with age). His CK was slightly above reference range (286 IU/L; normal range: 43 – 234 IU/L). The blood cell count showed macrocytosis, mild thrombocytopenia (178 x 10^3/uL; range 190-468 x 10^3/uL), monocytosis (1.8 x 10^3/uL; normal range: 0.075-0.85 x 10^3/uL) and lymphocytosis (3.6 x 10^3/uL; normal range:0.594 – 3.305 x 10^3/uL).

Diffuse spontaneous electrical activity was present on EMG evaluation of all appendicular muscles, axial muscles, in the muscles of mastication and the tongue. Spontaneous activity was characterized by prolonged insertional activity, fibrillation potentials and positive sharp waves and were most pronounced in the cervical musculature and muscles of mastication. Nerve conduction studies in the ulnar and tibial nerves demonstrated normal conduction velocity (52m/s and 59m/s) with reduced amplitude. The F wave, repetitive stimulation and cord dorsum studies were normal. The electrophysiological findings were consistent with a severe, generalized myopathy.

Grossly, biopsied muscles appeared pale and atrophied. Biopsies from the triceps brachii and vastus lateralis muscles were similar in appearance with numerous myofibers having the appearance of myotubes with diameters of <30μm and containing prominent central nuclei (Figure 1A). Type 1 fibers predominated (not shown). Myofibers had the appearance of necklace fibers [30] using the succinic dehydrogenase (SDH) reaction with prominent central blue staining and sub-sarcolemmal blue rings within the fibers (Figure 1B). No inflammation, necrosis, fibrosis, fiber loss, organisms or other specific cytoarchitectural abnormalities were obvious. Using immunofluorescence staining, an abnormal pattern was

found in muscle from the affected puppy compared to close to age-matched control muscle using antibodies against RYR1 (SR marker) and DHPR (t-tubule marker). The abnormal localization of the SR and t-tubule markers supported an abnormal pattern of triad structures (Figure 1C). These pathological and immunochemical changes were consistent with XLMTM. Western blotting demonstrated an absence of a myotubularin protein band (Figure 2).

The dog was discharged to the owners and remained on clindamycin until results of protozoal serology (which were negative) and muscle biopsy were available. Weakness progressed over the ensuing 4 weeks and the puppy was humanely euthanized at 5 months of age because of inability to swallow and aspiration of food and water when eating and drinking.

Information on the sire and dam as well as littermates from the proband's litter, 3 previous and 1 subsequent litters was available. Both sire and dam are reportedly clinically normal at time of writing. Overall the breeding pair produced 45 live and 2 still-born puppies. Of the live births, 18 were female (all survived to become healthy adults) and 27 were male, 11 of which died within the first month of life; the proband survived to 5 months due to intervention with assisted feeding. One of the female offspring subsequently produced 3 litters totaling 36 dogs, 21 females and 15 males, 8 of which died within 2 weeks of birth.

Whole genome sequencing of the proband as described above resulted in an average depth of coverage of 42x, with 99.3% of bases covered at least 5x. We detected 7,096,255 variants (biallelic and multialleic) in the affected dog. Of these variants, 53,710 were unique to the proband. These variants had 66,849 distinct variant effects contained within 7,857 genes. Among the variant effects, 38 were predicted by VEP to have a high impact on gene function, 241 a moderate effect, 232 a low effect, and 66,338 a modifier effect. A single base pair change that caused a premature stop codon was identified in exon 13 of the *MTM1* gene, (ChrX: 118903496 C > T, c. 1467C>T, p.Arg512X) and confirmed to be hemizygous in the male pro band by Sanger sequencing (Figure 3). On testing of the additional 17 Boykin spaniels for which DNA was available, the dam was heterozygous for the variant (and clinically normal), the sire was hemizygous for the reference allele, 3 male puppies were hemizygous for the variant and all died by 2 weeks of age. Three females were heterozygous and clinically normal, one of these heterozygotes was the female that was producing presumably affected males, and the remaining 8 dogs were homozygous wild type.

The mutant DNA sequence for exon 13 of *MTM1* was entered into ExPASy translate tool to confirm the predicted consequence, and we found that residue 512 (Arginine) was changed to a stop codon as expected.

4. Discussion

We have described the clinical, histopathological and genetic findings in a male Boykin spaniel with XLMTM. Onset of signs was at 3 weeks of age when the puppy showed difficulty in nursing, he presented with severe and progressive signs of neuromuscular

weakness (tetraparesis with muscle atrophy and markedly reduced segmental spinal reflexes) at 12 weeks of age and was euthanized due to difficulty swallowing at 5 months of age. Electrophysiological work up was consistent with a generalized myopathy, affecting the muscles of musculature of the head and cervical spine most severely, and histopathology on muscle biopsies revealed a centronuclear myopathy. Whole genome sequencing identified a single base pair change that caused a premature stop codon in *MTM1*, (ChrX: 118903496 C > T, c.1467C>T, p.Arg512X) that resulted in complete loss of myotubularin on Western blotting. The dam was confirmed to be a heterozygote and affected male siblings were hemizygous for the variant.

When compared with existing canine XLMTM, this mutation was associated with a very severe phenotype analogous to the signs that occur in infant boys. Affected puppies developed signs and died within a month of birth if given no supportive care. The proband examined in more detail here was given feeding assistance, aiding its survival for 5 months. This phenotype is slightly more severe than that reported for Labrador retrievers and Rottweilers. Clinical findings of muscle atrophy, tetraparesis and exercise intolerance, hypo- and areflexia and difficulty holding the head up reflect the human and canine phenotype. It is unusual for dogs to develop difficulty holding their head up due to the supportive role of the nuchal ligament, but it was notable that the EMG changes were extremely profound in the cervical spine. The early onset of difficulty feeding and the documented oropharyngeal dysphagia reflect involvement of the tongue and muscles of mastication and are particularly devastating for a young puppy that needs to nurse.

Histopathological abnormalities in skeletal muscle in spontaneously occurring canine XLMTM including those in Labrador Retrievers [19], Rottweilers [16] and the Boykin Spaniel of this report, in human patients, in the mouse model [12], and in the zebrafish model [9] are similar. These changes include variability in myofiber size and hypotrophic fibers containing prominent internal nuclei similar to fetal myotubes. Necklace fibers are considered a common feature of XLMTM in all species and should warrant a search for a mutation in *MTM1*. Necklace fibers are not a common feature of the related centronuclear myopathy (CNM) in Labradors associated with the *PTPLA* gene mutation although they have been reported[32]

Myotubularin is a lipid phosphatase that, through this function, plays a role in membrane trafficking. Pathogenic mutations in *MTM1* result in disorganization of triad structures and a failure in excitation-contraction coupling [9]. The myotubularin protein has 6 functional domains, GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularins), RID (Rac-induced recruitment domain), PTP (protein tyrosine phosphatase domain), SID (SET-interacting domain), PEST and PDZ-BS [33–36]. Mutations are dispersed across the entire gene, typically resulting in a complete loss of myotubularin production[11]. The stop codon introduced by the mutation in exon 13 in Boykin spaniels resulted in complete loss of myotubularin on Western blots and a severe phenotype with onset and death occurring within one month of age reported in many of the male offspring. Onset of signs is earlier than that reported in Labrador retrievers and Rottweilers (onset at 7 weeks in both breeds). The mutation in Labrador retrievers resulted in loss of myotubularin expression and Western blots were not performed in the Rottweilers. The reason for the younger age of onset in

Boykin spaniels is unclear at this time but there are numerous reported mutations throughout *MTM1* associated with human XLMTM, many of which result in myotubularin deficiency and cause a severe phenotype, comparable to the Boykin spaniels reported here [5,11,37,38].

5. Conclusions

We report here a family of Boykin spaniels with canine XLMTM due to a premature truncating codon in exon 13. The mutation causes a severe, fatal form of the disease in male puppies. The clinical signs and histopathology are comparable to human XLMTM and these dogs might serve as another canine model of this rare congenital myopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MTM1	myotubularin 1
XLMTM	X-linked myotubular myopathy
EMG	electromyography
SR	sarcoplasmic reticulum

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Highlights

- Boykin spaniels develop XLMTM due to a single base pair change.
- The mutation causes a premature stop codon in exon 13 of *MTM1.*
- Affected male puppies develop a severe, fatal phenotype.
- Histopathology shows necklace fibers and abnormal localization of triad structures.

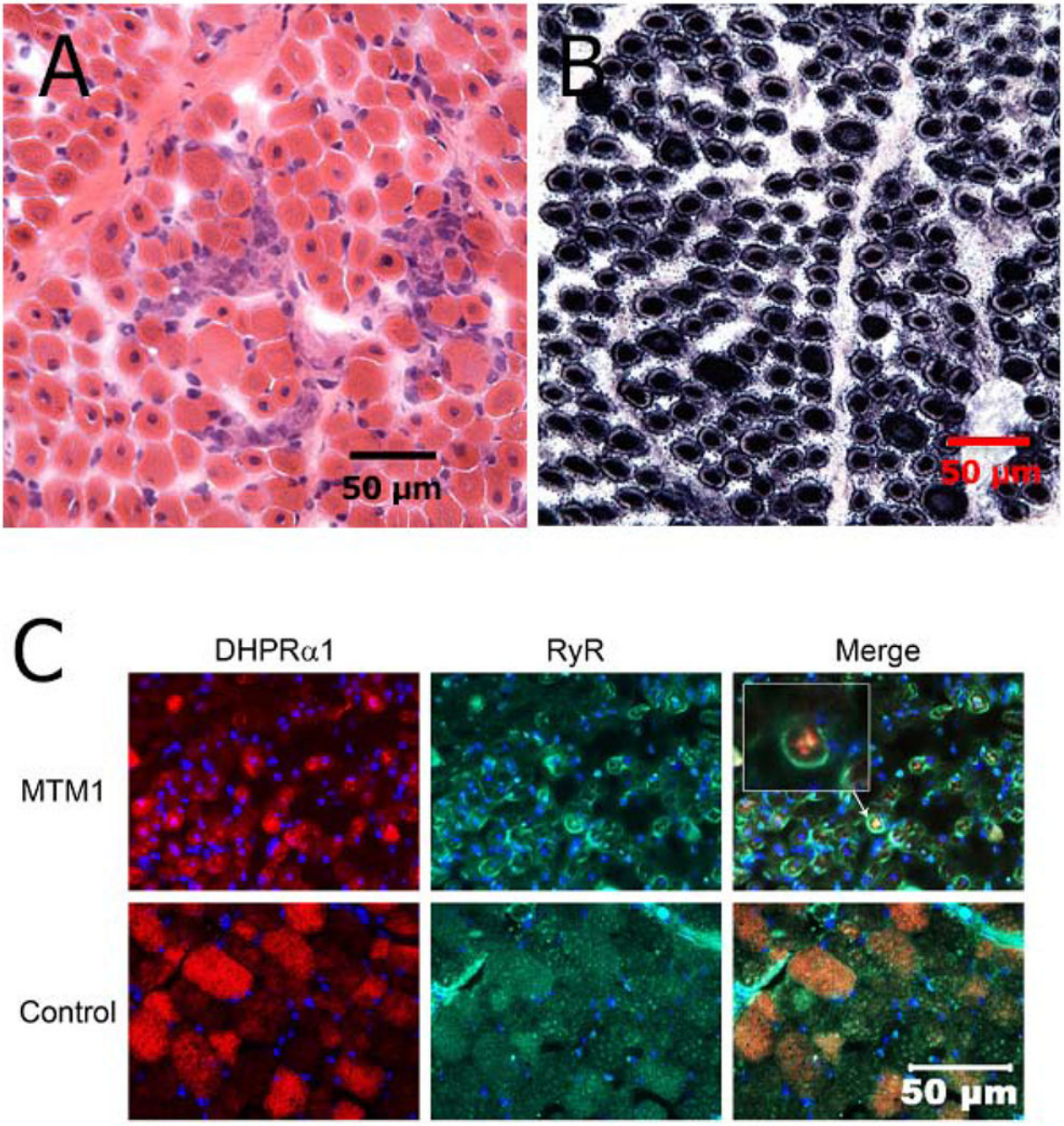

Figure 1.

Cryosections from the vastus lateralis muscle are shown using the H&E stain (A) and the succinic dehydrogenase reaction (B). Most myofibers were hypotrophic and many contained prominent large internal nuclei. As highlighted dark blue with the succinic dehydrogenase reaction for mitochondrial localization, dense central staining was prominent in most myofibers with obvious subsarcolemmal rings consistent with abnormal mitochondrial accumulations and the histological appearance of “necklace fibers”. Immunofluorescent stainings (C) for the triad markers DHPRa1 (T-tubule marker) and RyR1 (SR marker) in

cryosections from an affected puppy and archived close to age-matched control muscle. Abnormal staining patterns for the triad proteins were evident in the affected puppy compared to control muscle. Markers for T-tubules and surrounding SR were concentrated in irregular densities within numerous myofibers in muscle from the affected puppy but not in the control muscle. Scale bar represents 50 μm.

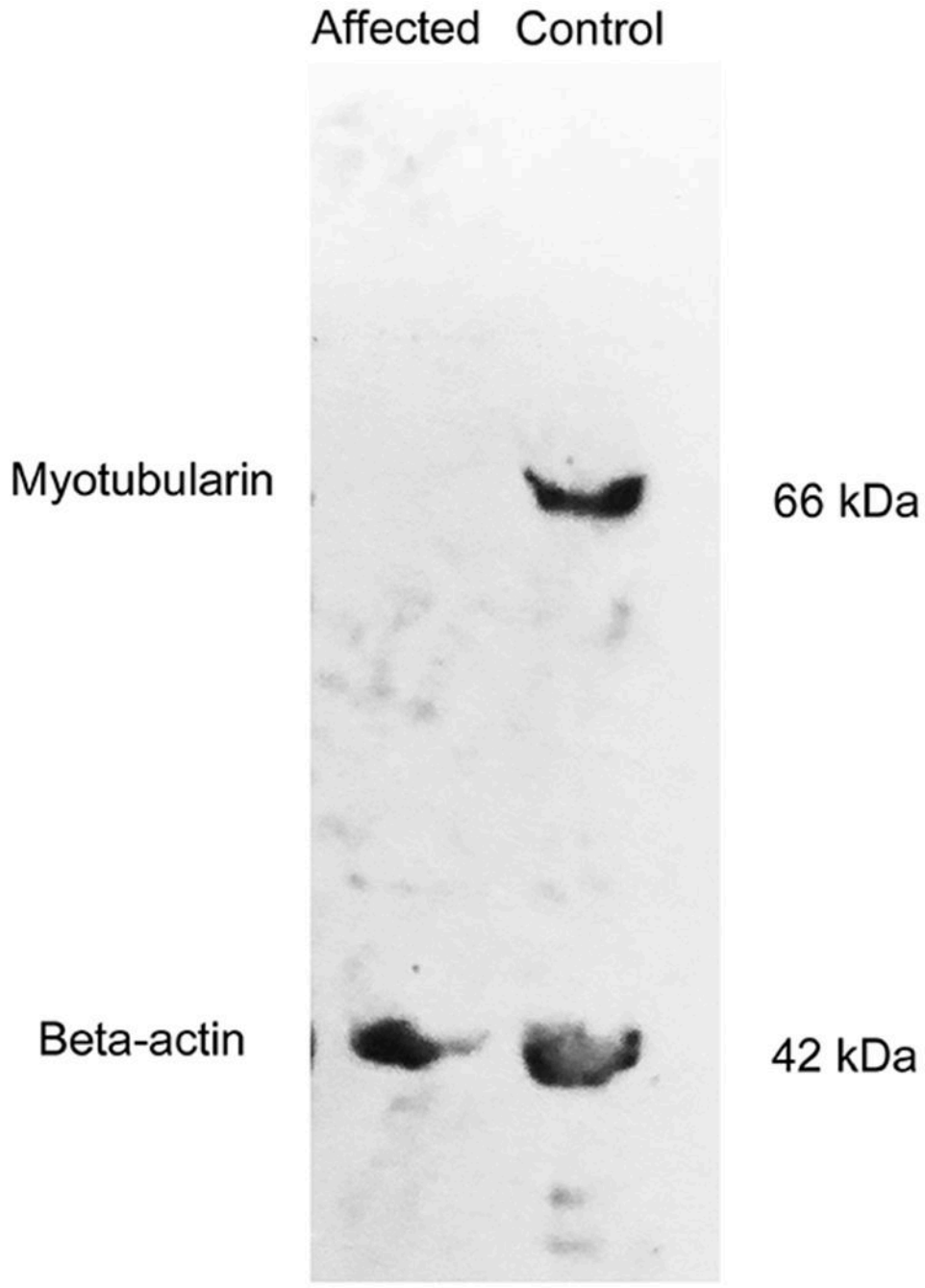

Figure 2.

Western blot of muscle extracts from the affected puppy and a close to age-matched control were stained for myotubularin and β-actin (loading control). A protein band was not detected using the myotubularin antibody in the affected puppy but was present in the control muscle. Staining of the band for β-actin was of subjectively similar intensity in both samples.

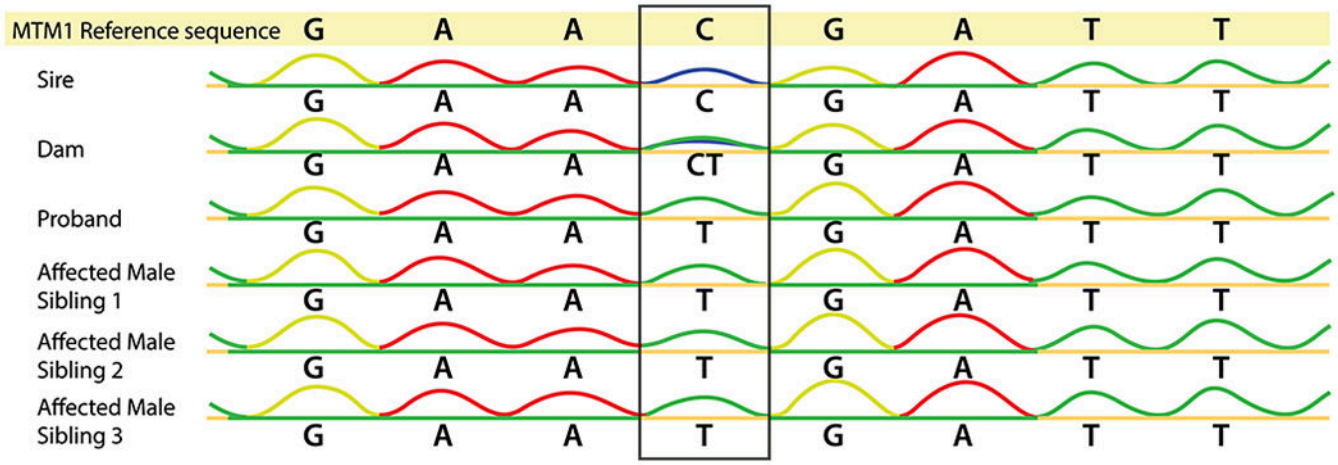

Figure 3:

Chromatogram showing results of Sanger sequencing of the sire, dam and 4 affected offspring including the proband. The C > T variant ChrX 118903496 is present in all affected male dogs and the dam is heterozygous for the variant.