MTM1 mutation associated with X-linked myotubular myopathy in Labrador Retrievers

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Mutations in the MTM1 gene encoding myotubulin cause X-linked myotubular myopathy (XLMTM), a well-defined subtype of human centronuclear myopathy. Seven male Labrador Retrievers, age 14–26 wk, were clinically evaluated for generalized weakness and muscle atrophy. Muscle biopsies showed variability in fiber size, centrally placed nuclei resembling fetal myotubes, and subsarcolemmal ringed and central dense areas highlighted with mitochondrial specific reactions. Ultrastructural studies confirmed the centrally located nuclei, abnormal perinuclear structure, and mitochondrial accumulations. Wild-type triads were infrequent, with most exhibiting an abnormal orientation of T tubules. MTM1 gene sequencing revealed a unique exon 7 variant in all seven affected males, causing a nonconservative missense change, p.N155K, which haplotype data suggest derives from a recent founder in the local population. Analysis of a worldwide sample of 237 unaffected Labrador Retrievers and 59 additional control dogs from 25 other breeds failed to identify this variant, supporting it as the pathogenic mutation. Myotubulin protein levels and localization were abnormal in muscles from affected dogs, and expression of GFP-MTM1 p.N155K in COS-1 cells showed that the mutant protein was sequestered in proteasomes, where it was presumably misfolded and prematurely degraded. These data demonstrate that XLMTM in Labrador Retrievers is a faithful genetic model of the human condition.

congenital myopathy | myotubulin | necklace fibers | canine myopathy | animal model

X-linked myotubular myopathy (XLMTM) is a well-defined subgroup of the centronuclear myopathies (CNMs) characterized by early onset and the presence of uniformly small muscle fibers with centrally placed nuclei resembling fetal myotubes (1, 2). Although centrally located nuclei can be found in many myopathies, clinical, genetic, and pathological factors can help distinguish these myopathies from XLMTM. Onset of clinical signs is typically at or near birth, and affected males have profound hypotonia and weakness accompanied by respiratory difficulties that usually require ventilatory support. The defective gene, MTM1, was identified in 1996 by positional cloning (3). Myotubulin, the protein encoded by the MTM1 gene, is a ubiquitously expressed phosphoinositide phosphatase implicated in intracellular vesicle trafficking and autophagy (4, 5). In skeletal muscle, myotubulin localizes to the triadic region, where it likely plays a role in lipid biogenesis or metabolism (6).

Animal models have played an important role in understanding the pathogenesis of how loss of MTM1 function leads to clinically evident myotubular myopathy. A classical knockout (KO) for the murine Mtm1 gene showed that myotubulin-deficient mice developed a progressive CNM during postnatal life that severely reduced life expectancy (7). Studies in this model, as well as in a related muscle-specific KO line, have demonstrated that myotubulin plays a role in muscle maintenance rather than maturation, and have confirmed that the primary tissue involved in XLMTM is muscle. Recently, a zebrafish model was created using morpholino antisense technology (8). Impaired motor function and centrally placed nuclei were similar to those seen in Mtm1 KO mice and XLMTM humans. Skeletal muscles of mtm1 morphants exhibited elevated levels of phosphoinositide-3-phosphate, a key substrate of myotubulin. Both morphology and human XLMTM patient muscle biopsy specimens exhibited abnormalities in triad organization, suggesting that myotubulin plays an important role in the development or maintenance of tubuloreticular network structure and function. Recently, similar morphological abnormalities in T tubules have been identified in the Mtm1 KO mouse model, accompanied by reductions in transcript and protein levels for several key excitation-contraction coupling proteins, including type 1 ryanodine receptors, as well as depressed sarcoplasmic reticulum (SR) Ca2+ release, demonstrating that a defect in excitation-contraction coupling might be a primary cause of weakness and hypotonia in XLMTM (9).

Along with their use in studies of the pathogenesis and mechanisms of disease, animal models are critical in preclinical trials of promising new drugs and therapies (10). Results of preclinical therapeutic trials in rodent models cannot always be directly extrapolated to the human condition, however. Thus, the identification and use of larger animal genetic homologs of human neuromuscular diseases is of increasing importance. Established models of X-linked dystrophin-deficient muscular dystrophy in Golden Retrievers (11, 12) and Beagles (13) have recently been used in therapeutic trials evaluating mesoangioblast stem cells (14) and systemic morpholino exon-skipping (15). Similarly, a colony of Labrador Retrievers with CNM due to a mutation in the Ptpla gene has been established in France (16–18); these dogs are being considered for upcoming preclinical trials. In a recent case report, we described a novel canine inherited myopathy presenting with progressive weakness and muscle atrophy beginning in the first few months of life (19). Here we describe this disorder in two kindreds of Labrador Retrievers and demonstrate
that these dogs represent a new genetic homolog of XLMTM with a missense variant in the MTM1 gene.

Results

Clinical Features of X-Linked Myopathy in Labrador Retrievers from Saskatchewan, Canada. Recently, we reported an apparently novel inherited myopathy affecting a 5-mo-old male Labrador Retriever who presented to the Western College of Veterinary Medicine (19). This puppy suffered from a 4-wk progressive course of weakness and muscle atrophy, leading to euthanasia at the end of the fourth week (Fig. 1A). Four male littermates developed similar clinical signs between 12 and 16 wk of age and were euthanized. Over the course of the intervening 3 y, five male Labrador Retrievers from two additional litters were referred to Western College of Veterinary Medicine with clinically similar presentations, and another affected male was evaluated at Wake Forest Medical Center.

Affected puppies were considered by their owners to be normal until they were 10–19 wk of age. However, three pups from kindred II were reported to be of small stature with muscle atrophy and pelvic limb weakness at the time of vaccination at 7 wk of age. Affected dogs exhibited pelvic limb weakness that progressed to an inability to rise or walk unassisted. Dogs that could stand exhibited kyphosis with a ventroflexed neck and walked with a short, choppy stride, collapsing after a few steps (Fig. 1A). All dogs had severe generalized muscle atrophy but no apparent muscle pain. Proprioception, as assessed by knuckling and hopping, was normal in all dogs that were able to move their limbs. Patellar reflexes were absent in all dogs, while withdrawal reflexes and cranial tibial reflexes were maintained in moderately affected dogs but absent in severely affected dogs. Dysphagia, a hoarse bark, and weakness of the muscles of mastication, leading to a dropped jaw, were noted terminally in all dogs. Dogs were non-ambulatory by 3–4 wk after the initial veterinary presentation. All dogs were euthanized at 15–26 wk of age.

Affected dogs came from three different litters that were initially thought to be unrelated; however, further investigation revealed that a female sibling of the dam of proband I.a was the maternal grand-dam of the independently ascertained proband I.e. Insufficient pedigree details are available for the third litter (kindred II in Fig. 1B) to prove or disprove a relationship. Inspection of the pedigrees clearly suggests that the myopathy in these dogs is inherited as an X-linked trait (Fig. 1B). All of the affected dogs evaluated were male, and each had multiple affected male siblings and only clinically normal female siblings. The presence in kindred I of affected male cousins whose dams were unaffected female siblings also clearly supports an X-linked pattern of inheritance.

Muscle Biopsies Show “Necklace Fibers” and Exhibit T Tubule Disorganization. Cryostat sections of muscle biopsy specimens from all seven studied affected male Labrador Retrievers showed similar morphologic abnormalities that were not evident in unaffected carrier littermates or normal control dogs (Fig. 2). Excessive variability in myofiber size was present (Fig. 2A and C), with a type 1 fiber predominance evident on ATPase staining. Many small fibers contained centrally placed nuclei resembling fetal myotubes; triceps biopsies from four affected males contained 5%,

![Fig. 1.](image)

**Fig. 1.** (A) A 4-mo-old male Labrador Retriever (dog I.a) affected with XLMTM illustrating the generalized muscle atrophy and characteristic kyphosis and ventroflexion of the neck indicative of generalized muscle weakness. (B) Pedigrees and genetic status of affected Labrador Retrievers from Saskatchewan, Canada. In the five litters depicted, only males were affected, supporting an X-linked recessive mode of inheritance. Three apparently unrelated probands (arrows) were eventually linked to two identifiable kindreds (I and II). All affected males (■) are related through carrier females (circles with red dots). Males marked with an asterisk were all genetically confirmed to be hemizygous for the MTM1 c.465C>A mutation, and females with red dots are confirmed heterozygous carriers. “CNM clear” indicates dogs whose DNA tested negative for the PTPLA SINE insertion mutation that causes canine CNM. Numbers within or below male or female symbols indicate the number of additional pups of a particular gender and affection status as inferred from their owners’ testimony. Not shown are two unaffected male and two unaffected female littermates of proband I.e and unknown additional unaffected littermates of dams I.b and I.c.

![Fig. 2.](image)

**Fig. 2.** Skeletal muscle histology in an affected male, I.e (A, C, and E), and unaffected carrier female, I.f (B, D, and F), whose muscle exhibited no detectable abnormal findings. H&E-stained fresh frozen muscle biopsy sections from the triceps (A and B) reveal extensive fiber size variation with numerous small, centrally nucleated myofibers resembling fetal myotubes characteristic of XLMTM, in the affected male (A). The vastus lateralis muscles (C–F) consistently exhibited similar but less extreme pathology. Numerous “necklace” fibers were highlighted with the oxidative stain NADH-TR (E and F). (Scale bar: 100 μm.)
6%, 15%, and 37% myofibers with central nuclei, respectively, compared with 4% in the vastus lateralis from the same four animals. A female carrier was indistinguishable from unaffected animals, with <1% centrally nucleated myofibers in both muscles. Subsarcolemmal ringed and central dense areas, so-called “necklace fibers” (20), were revealed with the oxidative reactions NADH-TR (Fig. 2E), succinic dehydrogenase, and cytochrome C oxidase. Necklace fibers were not observed in similarly stained sections from unaffected littermates or pups with CNM caused by PTPLA mutation (18). Immunofluorescence staining of myofibers from affected dogs using antibodies against markers for T tubules (DHPRx1) and SR (RYR1) revealed an abnormal localization of both structures, with T tubules and surrounding SR concentrated in irregular densities within numerous myofibers (Fig. 3A), but in none of an unaffected littermate’s myofibers. Immunostaining of affected dog muscles also revealed an abnormally condensed pattern of myotubulin staining in some myofibers that colocalized with the T tubule marker DHPRx1 (Fig. 3B). The frequency and quality of these fibers was reminiscent of the necklace fibers detected with oxidative stains, suggesting abnormal retention of myotubulin in these fibers. Immunostaining for dystrophin, spectrin, alpha sarcoglycan, dysferlin, caveolin 3, and utrophin was normal in muscles from affected dogs.

Central Nuclei, Skeletal Muscle Degeneration, and Loss of Triad Structures in Affected Dog Muscles. Recent studies have revealed structural and functional defects of the triads and excitation–contraction coupling in humans with XLMTM, as well as murine and zebrafish models of myotubulin deficiency (8, 9). Ultrastructural studies of affected dogs confirmed the centrally located nuclei with surrounding mitochondria (Fig. 4A). Myofibrillar disarray (Fig. 4B, D, and E) with and without mitochondrial accumulations, and perinuclear (Fig. 4C) and centrally located (Fig. 4D and E) whirled membranous structures similar to that seen in other species. Cristae-like structures (Fig. 4E) were evident within some membranous whorls, suggesting a mitochondrial origin of these abnormal structures. Triads were infrequent and disorganized in affected dogs (Fig. 4F) compared with a control littermate (Fig. 4G). The average number of morphologically normal triads per field was 2 ± 2 (range, 0–6) in affected pups, compared with 18 ± 9 (range, 6–32) in unaffected controls (P = 0.0004).

Identification of a Novel MTM1 Gene Mutation in Affected Dogs. Genetic analyses of the first affected dog was negative for both the SINE exonic insertion mutation in PTPLA known to cause autosomal recessive CNM and the X-linked DMD gene mutation responsible for Golden Retriever muscular dystrophy (19). Because the histopathological findings were similar in the affected dog and human XLMTM, we sequenced all 15 exons of the canine MTM1 gene. A C-to-A transversion in exon 7, causing the change c.465C>A, was present in two affected male dogs and in one obligate male carrier (Fig. 4F). This mutation (G155A) was not detected in any of the unaffected controls. Further, the mutation segregated with affected status. Of note, the mutation affected a conserved base in all species sequenced (C155A in Rattus norvegicus, C175A in Mus musculus, C165A in Canis lupus familiaris, C164A in Bos taurus, C165A in Equus ferus caballus, and the equivalent base in Homo sapiens in the fourth intron of the MTM1 gene). In addition, this mutation affected a highly conserved protein region between species (Fig. 4F).

![Image](https://example.com/image1.png)

**Fig. 3.** (A) Indirect immunofluorescence analysis of biopsies from the vastus lateralis muscle of two male Labrador Retrievers affected with X-linked myotubular myopathy (dogs II.d and I.e in Fig. 1A) and an unaffected carrier female (I.f in Fig. 1A) whose muscle histology and immunohistochemistry were indistinguishable from normal. Biopsy specimens from affected males revealed abnormal distribution of the SR and T tubule markers, ryanodine receptors (RYR1) and dihydropyridine receptors (DHPRx1), in numerous myofibers that exhibited subsarcolemmal and internal localizations; this pattern was not seen in myofibers from the unaffected littermate (I.f). (B) Indirect immunofluorescence analysis of the triceps muscle from dog I.e (Fig. 1B) showing apparent colocalization of a triad marker (DHPRx1 antibody) and myotubulin (R2867/C-terminal antibody) in abnormal distributions consistent with occurrence of necklace fibers. Staining of muscle from carrier I.f (bottom row) is indistinguishable from that in control biopsies. All panels are the same magnification (scale bar: 50 μm) except the magnified images of I.e in row 5 (scale bar: 10 μm).

![Image](https://example.com/image2.png)

**Fig. 4.** Ultrastructural studies of a Labrador Retriever (I.e in Fig. 1A) with XLMTM (A–F) and an unaffected female carrier littermate (I.f in Fig. 1B) showing centrally located nuclei (A), myofibrillar disarray (B–E) with or without mitochondrial accumulation, and whirled membranous structures (C–E). Triads were infrequent and in disarray in the affected dog (F, arrow and inset showing higher-power view) compared with the unaffected littermate (G, arrow and inset showing higher-power view). (Scale bars: 3.6 μm for A, 2.2 μm for B and G, 3.0 μm for C, and 1.8 μm for E and insets.)
ligate carrier at the heterozygous state, compared with several unaffected Labrador Retrievers as well as the canine (Boxer breed) reference sequence XM_850116.1 (Fig. 5B). This mutation results in a nonconservative change from asparagine to lysine, p.N155K, at a residue located in the linker region between the GRAM-PH and phosphatase domains (Fig. 5A and C). Subsequently, seven affected males, representing each of the affected families, were found to be hemizygous for this change, whereas three obligate female carriers were all heterozygous (Fig. 1B). To determine whether the association of MTM1 c.465C>A with canine congenital myopathy was absolute, we used both direct genomic PCR and DNA sequencing, along with a rapid 96-well plate–based TaqMan assay, to screen for this change in a panel of control canine DNA samples. The change was not seen in any of 237 unrelated and unaffected Labrador Retrievers from throughout North America, Europe, and Australia, nor was it detected in any of 59 additional control dogs from 25 other breeds.

Abnormal Expression of p.N155K Myotubularin in Vivo and In Vitro. Western blot analysis of myotubularin in skeletal and cardiac muscles of affected dogs revealed a profound loss of immunoreactive protein using two antibodies directed against the N- or C-termini of myotubularin (Fig. 6A and B). Muscle from WT and Mtm1 KO mice was used as a control to demonstrate antibody specificity. These studies showed greatly reduced myotubularin protein in extracts from either the quadriceps muscle or body section containing GRAM-PH (Fig. 6C–H), and additional studies revealed that these structures also labeled positively for polyubiquitinylated proteins, but were negative for markers of microtubules (beta-tubulin), Golgi apparatus (golgini-97), lysosomes (Lamp-1), early endosomes (EEA1), and endoplasmic reticulum (calnexin) (Figs. S1 and S2). A similar punctate staining pattern of aggregated proteins has been observed after expression of a catalytically inactive mutant of myotubularin-related protein 3 (22), suggesting that this may be a common consequence of destabilizing mutations of this family of proteins.

Transcript Analysis of MTM1 in Affected Skeletal Muscles. To determine whether the reduction in detectable myotubularin protein in skeletal muscles is more likely related to the effects of c.465C>A on mRNA or on protein stability, we performed rtPCR and DNA sequence analysis on MTM1 transcripts in control and affected canine skeletal muscles. mRNA from muscles of three affected males and one carrier female yielded robust PCR products of similar size and quantity compared with normal control RT-PCR products. DNA sequence analysis demonstrated that affected animals expressed only the mutated mRNAs, whereas muscle from the carrier female contained both
matches the human condition for size and scale, or for the indolent course that sometimes characterizes XLMTM in children. Here we report a canine homolog of XLMTM that reproduces many of the important clinical and pathological characteristics of human XLMTM in a larger species of similar scale to humans. Labrador Retrievers with mutated myotubularin are clinically normal at birth yet begin to exhibit progressive generalized muscle weakness and atrophy beginning as early as 7 wk of age. Without the option of intensive supportive care, such as intubation for respiratory support and gastrostomy tube feedings, which are common management strategies for human patients (23), affected dogs invariably require euthanasia between 3 and 6 mo of age. This differs from human XLMTM, in which patients are severely affected at or near birth but the myopathy, although often quite severe, is relatively nonprogressive (2). In this regard, canine XLMTM is similar to the mouse Mtmt1 KO, with clinical signs first appearing about 3–4 wk of age and leading to death by 7–9 wk of age (7). This apparent discrepancy between clinical courses in humans versus the mouse and canine models leads us to hypothesize that the progressive aspects of the human condition have manifested by birth and that the extended survival is a consequence of sophisticated supportive medical management available in modern medical centers. We postulate that differences in onset of the disorder might be due to variability in the timing of muscle differentiation among species, given that differentiation ends at about 16 wk gestation in human babies (24) and complete at birth in the mouse (7), and ends by approximately 4 wk postpartum in the dog (24). In zebrafish myotubularin morphants, the muscle phenotype is present at a very early time point (8), probably also reflecting rapid and compacted development in this species.

Histopathological abnormalities in skeletal muscle of XLMTM Labrador Retrievers (Fig. 2) are similar to those of XLMTM in human patients and the mouse (7) and zebrafish (8) models, showing excessive variability in myofiber size and variable numbers of myofibers containing prominent internal nuclei similar to fetal muscles. Necrosis fibers (20) were a common finding in muscle biopsy specimens from affected male XLMTM Labrador Retrievers, with characteristic subsarcolemmal rings and central changes highlighted with the oxidative reactions NADH-TR, succinic dehydrogenase, and cytochrome C oxidase. The carrier females available for study were all clinically unaffected, and a muscle biopsy from one exhibited no abnormalities. Although the necklace fibers have been recently described as a new histological marker of late-onset Mtmt1-related CNM, they also have been seen consistently in Mtmt1 KO mice (7, 9), and it has been suggested that recognition of necklace fibers in muscle biopsy specimens should be followed by Mtmt1 screening irrespective of the patient’s age and gender (20). Our results demonstrating necklace fibers in early onset canine XLMTM, but not in Ptppla-related canine CNM (17, 18), support this suggestion. Molecular and pathological studies of Mtm1 KO mice and transient knockdown zebrafish have recently revealed a critical role for myotubularin in establishing and/or maintaining structural and functional integrity of the membranous structures at the triads (8, 9). A striking ultrastructural finding in dogs with XLMTM, in addition to central nuclei, myofibrillar disarray, and whirled membranous structures, was the structural abnormalities of the T tubule network. Triads and adjacent SR from a dog with XLMTM were significantly dilated, disorganized, and decreased in number (Fig. 4 F and G) compared with an unaffected littermate. These findings also were supported by abnormal localization of SR and T tubule markers using immunohistochemical staining (Fig. 3). Given that triad abnormalities were recently reported in skeletal muscle from zebrafish myotubularin morphants (8) and Mtm1 KO mice (9), we suggest that altered phosphoinositide regulation in our canine model likely also affects triad organization or maintenance.

As of January 2010, the Leiden Open Variation Database of human Mtmt1 gene mutations (http://www.lodv.nl/mtmt1) listed 392 public entries representing 235 unique variants, of which 210 are considered pathogenic mutations associated with XLMTM.
There is no record of a missense mutation of p.N155 in a human patient; the nearest missense mutation is p.E157K, which was found in a family with unusually mild XLMTM (25). Although myotubularin protein was not examined in this case report, the extremely mild nature of the patients’ conditions strongly suggests that their muscle contained residual and partially active myotubularin (26, 27). In contrast, the dramatic reduction in myotubularin protein on Western blot analysis of affected dogs’ skeletal muscles, along with the mislocalization and aggregation of the mutated protein in proteasomes of transfected cells and in what might be necklace myofibers in affected dogs, suggests that the p.N155K mutation, which is in a linker region between the PH-GRAM and phosphatase domains, destabilizes the protein, leading to aggregation and premature degradation. This is consistent with the observation that most human missense MTM1 mutations tested by Western blot analysis result in significant reduction in myotubularin levels (28). The recessive nature of the mutation and lack of overt pathology in carrier females suggests that the pathogenic mechanism of this mutation involves a loss of function, which is also consistent with the observation of greatly decreased overall protein levels. Vertebrate myotubularins are all cellolinear and highly conserved, and p.N155 is invariant in all myotubularins examined from human to Xenopus (Fig. 5D); however, it is not conserved in the other myotubularin-related proteins (Fig. 5E), suggesting a critical and specific role for this residue in maintaining the correct spatial relationships between the PH-GRAM and phosphatase domains of myotubularin.

The genetic haplotype data demonstrating a recent local origin for the MTM1 p.N155K mutation suggest that Labrador Retrievers and other canine breeds from other parts of the world are unlikely to be at significant risk for this particular mutation. However, the recurring nature of XLMTM mutations in human populations also suggests that canine XLMTM likely has occurred independently before and is likely to recur in the future. As in humans, veterinarians and veterinary pathologists should consider an MTM1 mutation as a possible cause of any unexplained progressive myopathy in dogs, especially when necklace fibers or excessive central nucleation are prominent features of the myopathy. Canine XLMTM is an excellent model for the human condition, representing the only known large animal model available for preclinical trials of potential therapies. As such, these animals represent an invaluable resource that might be instrumental in developing effective treatments for this severe human congenital myopathy.

Materials and Methods

Animals. Over a 3-y period after the report on the original litter (19), five additional male Labrador Retrievers of age 14–26 wk, from two apparently unrelated litters were evaluated for generalized weakness and muscle atrophy at the Veterinary Medical Teaching Hospital, University of Saskatchewan. A fourth litter, whelped at Wake Forest University Medical Center, was also evaluated and included one additional affected male. All dogs tested negative for the known CNM mutation in the PTPLA gene (18). Complete blood counts and routine serum biochemistry profiles were unremarkable. Serum CK activities were normal or only mildly elevated. Electromyography was performed under general inhalational anesthesia in three of the dogs, showing abnormal spontaneous activity in several muscle groups. Muscle biopsy specimens were collected from multiple sites, including the lateral head of the triceps and vastus lateralis muscles in all five dogs. All animal studies were conducted during the course of routine clinical care under the auspices of the University of Saskatchewan’s Animal Care Committee.

Experimental Details. Experimental details of histopathology, histochemistry, and immunofluorescence staining; immunoblotting; electron microscopy and quantification of triad structures; DNA extraction and MTM1 gene sequencing; rapid TaqMan assay for identification of an MTM1 mutation; SNP selection and genotyping of dogs; haplotype analysis; and transfection and subcellular localization of cDNA constructs are available in SI Materials and Methods.

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