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A Novel Mutation in CLCN1 Associated with Feline Myotonia Congenita

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Abstract

Myotonia congenita (MC) is a skeletal muscle channelopathy characterized by inability of the muscle to relax following voluntary contraction. Worldwide population prevalence in humans is 1:100,000. Studies in mice, dogs, humans and goats confirmed myotonia associated with functional defects in chloride channels and mutations in a skeletal muscle chloride channel (CLCN1). CLCN1 encodes for the most abundant chloride channel in the skeletal muscle cell membrane. Five random bred cats from Winnipeg, Canada with MC were examined. All cats had a protruding tongue, limited range of jaw motion and drooling with prominent neck and proximal limb musculature. All cats had blepharospasm upon palpebral reflex testing and a short-strided gait. Electromyograms demonstrated myotonic discharges at a mean frequency of 300 Hz resembling the sound of a ‘swarm of bees’. Muscle histopathology showed hypertrophy of all fiber types. Direct sequencing of CLCN1 revealed a mutation disrupting a donor splice site downstream of exon 16 in only the affected cats. In vitro translation of the mutated protein predicted a premature truncation and partial lack of the highly conserved CBS1 (cystathionine β-synthase) domain critical for ion transport activity and one dimerization domain pivotal in channel formation. Genetic screening of the Winnipeg random bred population of the cats’ origin identified carriers of the mutation. A genetic test for population screening is now available and carrier cats from the feral population can be identified.

Introduction

Myotonia is defined as delayed relaxation of voluntarily or reflexively contracted muscle [1] and is due to repetitively firing muscle action potentials after a single stimulus. Individuals affected with various forms of myotonia typically describe a painless, muscle stiffness that remits with several repetitions of the same movement; the so-called ‘warm-up’ phenomenon [2,3]. In some cases, however, the myotonia does not begin until one or two sets of the same movement are completed, termed delayed myotonia. If symptoms worsen with repeated exercise, paradoxical myotonia is more appropriate, which is usually due to mutant sarcolemmal Na+ channels [4].

In humans, myotonia congenita (MC) is characterized by mutations within CLCN1, a gene [5] encoding the skeletal muscle voltage-gated chloride channel CIC-1. This chloride channel is responsible for up to 80% of the resting sarcolemmal conductance [4,6,7] and belongs to the CIC family of anion channels (CIC-0, CIC-1, CIC-2 and CIC-Ka/Kb) and anion/proton antiporters (CIC-3–CIC-7) [8]. The channel architecture results in two largely independent protopores of CIC channels that are opened and closed by two structurally distinct gating processes [9]. In humans, CLCN1 is organized into 23 exons [10] and each allelic gene product is thought to contribute to its own pore. The structure of the chloride channel protein was elucidated with electron microscopy [11] and X-ray crystallography [12,13] following the discovery of bacterial homologues from Escherichia coli and Salmonella typhimurium. The CIC dimer consists of a transmembrane (TM) and a cytosolic cystathionine beta-synthase (CBS) domain comprising 23 alpha-helices (A-V) and 5 beta-strands. CLCN1 functions as a homodimer with four proposed sites of dimerization [12,14].

MC shows autosomal dominant or recessive patterns of inheritance with varying degrees of severity. A correlation between the severity of the phenotype and the mode of inheritance has been suggested; the autosomal dominant (Thomsen Disease, OMIM 160800) form associated with a milder phenotype and the recessive form (Becker Disease or recessive generalized myotonia, OMIM 255700) associated with severe myotonia [15,16]. The disease phenotype is evident when the net chloride conductance is below 50% of normal, thus usually dominant-negative interactions that disrupt the channel are more
likely associated with autosomal dominant pattern of inheritance [2,3]. Mutations that cause early truncations of the protein are thought to present as an autosomal recessive mode of inheritance, due to the loss of the ability to form dimers [17,18].

In veterinary medicine, MC has been described in several mammalian species. The goat provided the first opportunity to define the cellular defect in myotonic muscle [19,20], as muscle fibers from myotonic goats exhibited greatly diminished chloride conductance [21]. In this first molecular model, Beck et al. (1996) reported a missense mutation affecting the carboxy terminus of CLCN1 that reduces the channel open probability at physiologic voltages. In goats, MC is inherited as an autosomal dominant trait [22]. Since the first description of caprine MC [23], historical, clinical, electrodiagnostic and histopathologic features have become well-established in horse [24], dog [25,26] and cat models [27–30]. In the feline model, historical and clinical findings, along with routine blood testing, muscle percussion, cardiac examination, electrodiagnostic testing and muscle histopathology have been characterized in two related kittens and in four kittens from separate litters [21,23]. Both reports speculated that the disease was inherited as an autosomal recessive trait.

In the present study, feline MC from a feral random bred colony was characterized with respect to routine clinical investigations, skeletal muscle histology and immunohistochemistry, and electrodiagnostic findings. Direct sequencing of the candidate gene, CLCN1, and analysis of mRNA from muscle biopsies were conducted in the cats.

Results

Clinical presentation

Abnormalities on general physical examination common to all five affected cats included a restricted jaw opening, halitosis, varying degrees of gingivitis, hypothyroidism, marked dental calculus accumulation with palpable loose teeth and evidence of poor grooming habits. Severe muscle hypertrophy was found along the cervical spine, proximal aspects of all limbs (Video S1) and the tongue, which protruded from the mouth (Figure 1 and Video S2). One cat showed signs of respiratory distress (stridor, open mouth breathing) upon examination that lasted for several minutes. Serum creatine kinase (CK) activity was mildly elevated in one of the five cats tested.

All affected cats had a normal mentation. On cranial nerve examination, abnormalities were limited to blepharospasm after testing both the menace responses and the palpebral reflexes (Video S2). The gait was stiff and short-strided in all limbs, especially in the pelvic limbs, accompanied by a decreased ability to adduct all limbs while walking. While ambulating, four cats would intermittently assume a frozen posture in whatever degree of flexion and/or extension the joints were in at the given instant, and would remain static for several seconds before resuming ambulation (Video S3). No obvious ataxia was observed. In one cat, a plantigrade stance was obvious. Upon postural reaction testing, proprioceptive positioning and hopping were normal in all limbs. Upon visual placing in the thoracic limbs, all cats showed a progressive worsening with this testing procedure, ultimately ending after several repetitions with absent visual placing in the thoracic limbs and active cervical ventroflexion. Abnormalities on spinal reflexes were limited to absent cutaneous trunci reflexes bilaterally in all cats. Percussion of the triceps muscle in one cat produced a prolonged dimpling of the muscle (Video S1). A startle response was not observed in these cats.

Electromyography, Nerve Conduction Velocity, Repetitive Nerve Stimulation, Muscle Biopsy and Cardiac Findings

Appendicular as well as axial skeletal muscle was tested. Prolonged insertional activity with myotonic discharges were identified in all skeletal muscles tested and showed the characteristic waxing and waning amplitude (Figure 2 and Video S4). The discharges had a mean amplitude of 277 μV (130 μV, 240 μV and 470 μV) and a mean frequency of 300 Hz (240 Hz, 260 Hz and 400 Hz). Because of the remarkably high frequency of the myotonic discharges, the sound generated from these discharges on the EMG loudspeaker resembled a swarm of bees.

Left tibial nerve motor conduction velocity was tested in two cats and found to be within normal limits. Supramaximal repetitive nerve stimulation was performed at the left distal hock in three cats at a repetition rate of 3 Hz. Decrement was observed in two cats tested, with percentage decreases in area under the curve between the 1st and 10th waveforms of 31.4% and 66.9% and percentage decreases of 48% and 67% between the 1st and 30th waveforms (Figure 3).

Myofiber size ranged from 30–150 μm (normal feline reference interval 40–50 μm) [31]. A normal mosaic pattern of muscle fiber types was present. Intramuscular nerve branches were normal in appearance. No inflammation, necrosis, fibrosis or other cytoarchitectural abnormalities were found (Figure 4).

Cardiac auscultation of all cats was normal. Thoracic radiographs were taken in two cats and echocardiograms were performed in four cats. Thoracic radiographs showed no evidence of heart enlargement or congestive heart failure. Two-dimensional echocardiography revealed several moderator bands in the left ventricle in all four cats evaluated. Left ventricular diastolic function was normal in all cats evaluated based on transmitral flow patterns, solumvicul relaxation times, and/or tissue Doppler mitral annular motion. No cardiac hypertrophy or chamber enlargement was noted in any of the cats evaluated. Atrial premature complexes of unknown origin were noted in one cat.

CLCN1 sequence analysis and genotyping

The entire CLCN1 coding sequence (GenBank accession no. KJ364151) and partial 5’ and 3’ UTRs were analyzed for six cats (Table 1) representing the five affected cats and one random bred control. In humans, CLCN1 has one isoform and the length of the coding region within the transcript is 2967 bp. In the cat, CLCN1 has 23 exons (Table S1), the boundaries were confirmed by genomic sequencing of the five cats used for the analyses of CLCN1, producing a 2,970 bp coding sequence (CDS) that

Figure 1. The hypertrophic tongue in a cat affected with myotonia congenita. The tongue is very enlarged and constantly protrudes from the mouth. doi:10.1371/journal.pone.0109926.g001
translates into 989 amino acids (Figure S1). The average CDS identity between humans and cats is 87.5% and at the protein level, CLCN1 in felines is 89.5% identical to humans. Seven of the seventeen identified polymorphisms are exonic mutations (Table 1). Two mutations in exon 18 result in an amino acid substitutions, however, they were not concordant with the disease phenotype. One of the ten intronic mutations segregated concordantly with the phenotype and affects an exon/intron splice site in affected cats. The c.1930+1G>T transversion altered the 5’ splice site at the junction of exon 16 and intron 16 (Figure 5a). The effect of this polymorphism was investigated at the RNA level (Figure 5b). All the affected cats showed the same haplotype across all the identified polymorphisms (Table 1). All the mutations were confirmed by the RNA transcript sequence obtained from muscle tissues of affected and wildtype cats. Interestingly, the identified mutation is associated with the upstream absence of exons 15 and 16 (Figure 6). Intron 14 sequence showed polymorphisms in the control cat only, in a heterozygous state, while the affected cat sequence was identical to the wildtype sequence. To exclude retrotranscription artifacts, the RNA experiment was conducted twice, beginning at the RNA isolation step. In silico translation of the altered transcript predicts the lack of 116 amino acids, from residues 557 to residue 643 of the protein (Figure S1). The predicted premature CLCN1 protein truncation would cause the partial absence of the first highly conserved CBS1 (cystathionine β-synthase) domain and the third dimerization domain p.578Y. The strength of the 5’ splicing sites (5’ss) was calculated for exon-intron 14, 15 and 17 boundaries using the Maximum Entropy Model score, the Maximum Dependence Decomposition Model score, the first-order Markov Model and the Weight Matrix Model score. The strength of the 5’ss in all the tested boundaries was similar in each method when...
compared, suggesting exon-intron 15 5’ss as the strongest. Complete donor splice site strength is confirmed in the mutated sequence (Table S2).

Within 244 cats screened for the c.1930+1G>T polymorphism (Table 2), the mutation was only found to be heterozygous in three cats from the Winnipeg feral population (n = 35). The cats pertaining to the 26 breeds (n = 182) as well as the two random bred populations (n = 14) all tested wildtype.

Immunohistochemical localization of voltage-dependent chloride (Cl-) channels

Cryosections (8 μm) from the biceps femoris muscle of affected and control cats were incubated with rabbit polyclonal antibodies against the C-terminus and N-terminus of CLCN1 and co-stained with a monoclonal antibody against dystrophin (marker for the muscle sarcolemma). The sections were then processed by indirect immunofluorescence. Staining using both antibodies against CLCN1 was localized to the interior of the myofiber and was distinctly punctate in nature without co-localization with dystrophin to the muscle sarcolemma (Figure 7). To further investigate the subcellular localization of the chloride channels, antibody markers for the sarcoplasmic reticulum (ryanodine receptor) and T-tubule system (dihydropyridine receptor DHPRα1) were co-stained with both antibodies against CLCN1 and compared to control muscle. Staining strongly suggested either co-localization or a close association with the T-tubule system (Figure 8).

Discussion

Voltage-dependent chloride (Cl-) channels are transmembrane proteins that represent Cl- permeation pores. Chloride channels are as abundant as cation channels (Na+, K+, Ca2+) and participate in many physiological tasks, including the maintenance of normal cellular excitability, the control of neurotransmitter release and the transport of ions across epithelial cells. MC was one of the first genetic diseases shown to be caused by mutations in genes encoding ion channels [5,32]. The aim of this paper was to describe the clinical characteristics of a cohort of cats with feline myotonia congenita.

Table 1. SNP analyses of CLCN1 in cats with and without myotonia congenita.

| SNP                  | Sample ID | Pheno | Position | Genotype | Mutation Type |
|----------------------|-----------|-------|----------|----------|---------------|
| Genotype             |           |       |          |          |               |
| E15.e1278G>A         | Cat1      | A     | 1278     | G        | Insertion     |
| E15.e1668C>T         | Cat2      | A     | 1668     | C        | Deletion      |
| E15.e1668C>T         | Cat3      | A     | 1668     | C        | Deletion      |
| E15.e1668C>T         | Cat4      | A     | 1668     | C        | Deletion      |
| E15.e1668C>T         | Cat5      | A     | 1668     | C        | Deletion      |
| E15.e1668C>T         | Control   | A     | 1668     | C        | Wildtype      |
| E15.e1668C>T         | Ensembl   | A     | 1668     | C        | Wildtype      |

*This polymorphism is investigated in detail by the authors.*

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Figure 5. Electropherogram representing the genomic and RNA sequence of \textit{CLCN1} in wild-type and myotonia congenita cats. a. Genomic sequence of three control cats and the five affected cats. The red rectangle indicates the c.1930+1G>T polymorphism in the first base of intron 16 associated with MC in the domestic cat. b. RNA sequence lacks exon 15 and 16, while no alterations are observed in the control cat at exon junctions 14–15 and 16–17. doi:10.1371/journal.pone.0109926.g005

Figure 6. Schematic representation of \textit{CLCN1} splicing in the wild-type cat and myotonia congenita cats. In exon 15, the yellow line represents the third dimerization site (dim site), while the blue box represents the CBS1 domain, present in exon 16 and 17. The black triangle represents the identified mutation and the two red circles connected by a red dashed line represent the donor splice site and the acceptor splice site in the mutated protein. a. Normal splicing occurring in the wild-type subject, both dimerization sites and CBS1 are present. b. In the affected cat, the mutation in intron 16 is associated with the absence of exons 15 and 16, and, therefore the protein lack the dimerization domain. doi:10.1371/journal.pone.0109926.g006
MC along with novel electrophysiologic findings and the molecular characterization of the mutation within the CLCN1 gene.

MC was characterized previously in a 5-month old intact male and in 9-month old intact female domestic shorthair littermates [20], as well as four closely related kittens from 2 related queens and unknown sires [29]. Although a mutation was not identified, both reports suggested the condition was a hereditary disease. Findings in common to both studies included an unthrifty appearance, a short-strided, choppy gait (more severe in the pelvic limbs), blepharospasm upon palpebral reflex testing or startling, dysphonia and prolonged dimpling of the tongue after percussion (percussion myotonia). The general physical examination and neurologic findings in the cats of this study are consistent with both previous reports. All cats from this feral breeding colony showed a protruding tongue with excessive drooling, prominent neck and proximal limb musculature, decreased range of jaw motion, oral disease, bilateral blepharospasm upon testing, a short-strided gait with decreased ability to adduct the limbs and a progressive decrease through successive attempts of visual placing in the thoracic limbs. This progressive decrease in the visual placing response was ascribed to transient weakness and no clinical significance was ascribed to the absent cutaneous trunci reflexes as this reflex has been previously shown to be sometimes absent in otherwise neurologically normal cats [62]. No clinical evidence of cardiac disease was detected in this study, consistent with previous reports of feline MC [28]. MC is a disease that typically does not affect either smooth or cardiac muscle [33].

Previously reported abnormal electromyographic findings in feline MC included increased insertional activity and myotonic discharges, with no evidence of defective nerve conduction. These findings were duplicated in all cats tested in the present study. In addition, the frequency of myotonic discharges reported herein was unique, with a mean frequency of 300 Hz. To our knowledge, this is the first report documenting such a high frequency of myotonic discharges. Classically, myotonic discharges occur with a frequency of 50–100 Hz and are described as sounding like a motor cycle revving or a dive bomber [1]. The higher frequency of myotonic discharges in the cat is likely the reason for the atypical sound in these cats, resembling a ‘swarm of bees’. Myotonic discharges that wax and wane are the characteristic electrophysiologic abnormality in MC. Diminished chloride conductance across the sarcolemma leads to increased input resistance such that the restricted extracellular space within the myofibers and decreased potassium accumulation in the transverse tubules [39]. It has been proposed that the decrement is the electrophysiologic correlate of weakness in some forms of MC. The weakness may be attributed to muscle depolarization secondary to sodium channel inactivation (depolarization block) at times of more extreme muscle depolarization, with motor axons subsequently firing at higher rates to control a weak muscle [39]. The degree of decrement, however, has not been shown to be associated with clinical severity [35]. A recent cohort study in humans has shown that decrement with the 5 Hz nerve stimulation protocol had a sensitivity of 66% for recessive MC with good reproducibility, whereas those patients with dominant MC or myotonia secondary to a defect affecting the skeletal muscle sodium channel showed no decrement at such a stimulation frequency. Decrement in recessive MC has been suggested to be related to variation in the CLCN1 alleles, including nonsense, splice site frame-shift and missense mutations [35–38].

In people with MC, non-specific histologic changes include abnormal variations in myocyte diameter, fiber hypertrophy, absence of type Ib fibers, central location of fiber nuclei and mitochondrial aggregates [40]. Variable findings have been described for both light and electron microscopy in feline MC, including occasional degeneration of individual myofibers associated with localized proliferation of sarcolemmal nuclei with few centrally located nuclei, moderate diffuse myofibril hypertrophy, rounding of the sarcoplasm, clear cytoplasmic vacuoles within many myofibers and mild dilatation of transverse T-tubules [27–29]. The mean myofiber perimeter and areas were previously measured and compared to age-matched controls using image software, showing increased perimeters and areas of myofibers in affected cats [27,29]. The muscle profiles in this study showed hyper trophy of both type 1 and type 2 fibers with a normal mosaic pattern of muscle fiber types and no evidence of inflammation, necrosis, fibrosis or other specific cytoarchitectural abnormalities, consistent with a diagnosis of MC.

In our study, results of immunofluorescent staining in both affected and control cats support localization of the chloride channels close to or at the location of the T-tubules. Immunofluorescent staining for chloride channels in both affected and control cat muscle did not support localization to the sarcolemma. Since staining was similar in both affected and control cats, the mutation identified in these myotonic cats likely results in impaired ion channel function rather than a mislocalization. However, additional more sophisticated studies that are beyond the scope of this study would be necessary to determine channel density or more precise localization.

In recent years, a large number of mutations associated with MC have been identified in CLCN1 in dog [41,42], goat [22], horse [43], water buffalo [44], mouse, [45] and human [5]. These mutations are dispersed throughout the entire coding and non-coding regions and result in distinct alterations of channel kinetics. Unexpected insights into the structure-function relationship are provided by the discovery of random sequence alterations that impair channel function, providing an alternative approach to identify elements in the sequence essential in channel function. The chloride channel consists of transmembrane domains, three dimerization domains and cystathionine β-synthase domains, critical for ion transport activity.
In the current study, **CLCN1** was analyzed in five cats with classical MC. Relatedness within the cats included in the study is not known, however, the cats were identified as originating from the same feral colony in Winnipeg, Canada. An intronic mutation in intron 16 leads to a splicing error, confirmed by the cDNA analysis, which translates to a lack of exon 15 and 16 and a 116 amino acids gap in the protein. Since the alternative splicing is atypical, the experiment was conducted from freshly isolated RNA and retrotranscription products. The same splicing variation was detected in both experiments, excluding retrotranscription artifacts as being responsible for the observed splicing. The entire sequence of intron 14 was also acquired and excluded possible intronic mutation associated with the detected splicing variant. The **c.1930+1G>T** appears to be the polymorphism associated with MC in the domestic cat.

**Table 2.** Domestic cats genotyped for the **CLCN1** mutation associated with myotonia congenita.

| Breed                  | Phenotype | Genotype | No. | G/G | G/T | T/T |
|------------------------|-----------|----------|-----|-----|-----|-----|
| Abyssinian             | Normal    |          | 9   | 9   | 0   | 0   |
| American short hair    | Normal    |          | 8   | 8   | 0   | 0   |
| Bengal                 | Normal    |          | 7   | 7   | 0   | 0   |
| Birman                 | Normal    |          | 7   | 7   | 0   | 0   |
| British short hair     | Normal    |          | 7   | 7   | 0   | 0   |
| Burmese                | Normal    |          | 7   | 7   | 0   | 0   |
| Chartreux              | Normal    |          | 7   | 7   | 0   | 0   |
| Cornish Rex            | Normal    |          | 8   | 8   | 0   | 0   |
| Devon Rex              | Normal    |          | 7   | 7   | 0   | 0   |
| Egyptian Mau           | Normal    |          | 7   | 7   | 0   | 0   |
| Japanese Bobtail       | Normal    |          | 7   | 7   | 0   | 0   |
| Korat                  | Normal    |          | 7   | 7   | 0   | 0   |
| Maine Coon             | Normal    |          | 9   | 9   | 0   | 0   |
| Manx                   | Normal    |          | 7   | 7   | 0   | 0   |
| Norwegian Forest Cat   | Normal    |          | 9   | 9   | 0   | 0   |
| Oicat                  | Normal    |          | 7   | 7   | 0   | 0   |
| Oriental               | Normal    |          | 7   | 7   | 0   | 0   |
| Persian                | Normal    |          | 7   | 7   | 0   | 0   |
| Ragdoll                | Normal    |          | 7   | 7   | 0   | 0   |
| Russian Blue           | Normal    |          | 7   | 7   | 0   | 0   |
| Siamese                | Normal    |          | 7   | 7   | 0   | 0   |
| Siberian               | Normal    |          | 7   | 7   | 0   | 0   |
| Sphynx                 | Normal    |          | 7   | 7   | 0   | 0   |
| Tonkinese              | Normal    |          | 7   | 7   | 0   | 0   |
| Turkish Angora         | Normal    |          | 7   | 7   | 0   | 0   |
| Turkish Van            | Normal    |          | 7   | 7   | 0   | 0   |
| Random Bred (Winnipeg) | Affected  |          | 5   | 0   | 0   | 5*  |
| Random Bred (France, USA)| Normal   |          | 35  | 32  | 3   | 0   |
| Total                  |           |          | 244 | 236 | 3   | 5   |

*Cats genotyped by direct sequencing.*

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In the current study, **CLCN1** was analyzed in five cats with classical MC. Relatedness within the cats included in the study is not known, however, the cats were identified as originating from the same feral colony in Winnipeg, Canada. An intronic mutation in intron 16 leads to a splicing error, confirmed by the cDNA analysis, which translates to a lack of exon 15 and 16 and a 116 amino acids gap in the protein. Since the alternative splicing is atypical, the experiment was conducted from freshly isolated RNA and retrotranscription products. The same splicing variation was detected in both experiments, excluding retrotranscription artifacts as being responsible for the observed splicing. The entire sequence of intron 14 was also acquired and excluded possible intronic mutation associated with the detected splicing variant. The **c.1930+1G>T** appears to be the polymorphism associated with MC in the domestic cat.

Splice sites (5’s) are the elements at the 5’ end of introns and are extremely diverse, as revealed by the thousands of different sequences that act as 5’s in the human transcriptome [46]. Most of the 5’s are recognized by base-pairing with the 5’ end of a small nuclear RNA (snRNA) called U1 [47,48]. To date, over 9000 sequence variants in the −3 to +6 region of the 5’s are recognized in humans as 5’s consensus [46]. The use or avoidance of 5’s could depend on other sites and is not necessarily an intrinsic property of any given sequence, as in the case of some sequences in β-globin where sequences that resemble 5’s were used when a natural 5’s was inactivated [49,50]. The mechanism is termed cryptic 5’s. Previous work had shown that a mutation within a 5’s might inactivate it or lead to the use of cryptic 5’s [51]. In the present work, an unusual alternative splicing that leads to the lack of two exons upstream of the mutation is presented. The 5’s strength analysis results exclude a significant difference in the strength of exon-intron 14 boundaries, excluding a gain of strength or the presence of a strong 5’s as possible mechanisms involved in exon 15 and 16 skipping. Moreover, the complete sequence of intron 14 reveals no mutations concordant with the phenotype and possibly responsible for the alternative splicing. A second hypothesis is that entire exons can be skipped when located in an internal RNA loop [52].
evidence is supported in numerous studies where secondary structures showed influence in 5’ss selection and subsequent splicing [53]. A striking example is provided by the MAPT exon 10 alternative splicing, whose inclusion levels are dictated by the efficiency of the 5’ss recognition, which is compromised by downstream intronic nucleotides responsible for a base-pairing structure [54]. The mutation occurring in intron 16 could be associated with the appearance of a pre-mRNA structure, such as loop, during the splicing event directly responsible for the lack of both exons 15 and 16, or indirectly affecting the performance of RNA-binding proteins and the rate of transcription.

The characterized alteration in CLCN1 is predicted to cause a lack of 116 amino acids in the protein, from position 557 to position 643. The alteration deletes part of the first CBS domain [55]. Mutations within the domain, critical for ion transport activity, are associated with recessive and dominant forms of MC [56]. Moreover, the deletion removes the third, highly conserved, dimerization domain. It is possible to hypothesize that the lack of one of the dimerization sites prevents the mutated allelic gene product from being incorporated in the dimer, although the mutated product is still incorporated into the membrane. A recessive mode of inheritance was also confirmed by the genotyping results, where the heterozygous cats were confirmed to be healthy. Carrier cats were only identified in the Winnipeg feral population, supporting the hypothesis that a novel mutation that arose in an intact feral cat and was spread amongst the local feline random bred population. A genetic test is now available and carrier individuals can be identified and sterilized to prevent further spread of the genetic alteration within the local population.

Myotonia congenita is one of numerous inherited diseases of skeletal muscle that have been defined at the molecular level in recent years and this disease model had provided cogent insights regarding muscle excitation and contraction. For the first time, MC in Felis silvestris catus was molecularly defined and associated with an early truncation of the CLCN1 protein, establishing the cat as a potentially valuable new animal model of this disease.

Materials and Methods

Sample collection

Private owners of affected and control cats were recruited to voluntarily participate in the study. All procedures were approved by the University of Missouri, Animal Care and Use Committee. Muscle biopsy samples were obtained from a privately owned cat after acquiring informed owner consent and done for routine diagnosis. Five affected cats privately owned were obtained from the same cat shelter in Winnipeg, Canada. The shelter obtained these cats from various sources, including local feral populations under population control. DNA samples from the Canadian feral cat population and from the control populations (26 breeds and random bred cats from US and around the world) were collected.
from the cats non-invasively using buccal swabs at cat shows, shelters and the owners’ homes (Table 2). DNA was isolated from buccal swabs using the DNeasy Kit (Qiagen).

Clinical description and Sampling
The five affected adult cats from Canada were brought to the Neurology service at the University of Missouri – Columbia, Veterinary Medicine Teaching Hospital for examination and diagnostic testing. A history of all cats was collected from the veterinarian who referred cats for diagnostic testing. All cats underwent both a general physical examination and a detailed neurologic examination, as well having complete blood counts and serum biochemistry panels. Urinalyses were assayed on two cats. Thoracic radiographs were performed on two cats. An echocardiogram was performed on four cats. All electrodiagnostic testing was performed using a Cadwell Sierra Wave machine (Kennewick, WA) with concentric (coaxial) needle electrodes while the cats were maintained under general gaseous anesthesia. Electromyography, nerve conduction velocities and repetitive nerve stimulations were performed within 10–15 minutes of induction of general anesthesia in three tested cats. Biopsies from the right biceps femoris muscle were collected under general anesthesia in three cats. The muscle biopsy was bisected: the first portion was placed in a saline-dampened gauze sponge followed by submission under refrigeration for histopathologic assessment (Comparative Neuromuscular Laboratory; University of California, San Diego). The second portion was transferred into RNA later (Qiagen, Valencia, CA). RNA was isolated using the Invitrogen RNA mini Kit (Invitrogen, Carlsbad, CA).

CLCN1 genomic analysis
The genomic analysis of CLCN1 was conducted on genomic DNA from seven cats, including, the five affected random bred cats from Winnipeg and two random bred controls available in the laboratory. The complete CDS (23 exons) of CLCN1 is publicly available (http://ensembl.org) and can be found on cat chromosome A2: 157,284,157–157,315,463 (assembly version Felis_catus_6.2). CLCN1 intron 14 was fully amplified using the SequalPrep long range PCR kit (Invitrogen) as per manufacturers recommendations and sequenced using internal primers (Table S1). The assay was optimized using DNA from five wild-type samples and five affected samples, and DNA concentration was normalized to ~15 ng/μl for each sample to mimic average sample concentration from buccal swab isolation. Heterozygous samples for the assay optimization were made by combining wild-type and affected DNA with a 1:1 ratio. Graph of melting curves from the optimization are shown in Figure S2. A two-step cycling protocol consisting of an initial denaturation of 95°C for 5 min followed by 40 cycles of 95°C for 10 s and a combined annealing/extension step at 58°C for 30 s was carried out. The samples were subsequently subjected to a 65°C isotherm step for 90 s followed by a temperature gradient from 65°C to 85°C heating 0.1°C per step with 2 s wait at each step. Each genotype was analyzed in duplicate. The genotype was assigned using the Rotor-Gene ScreenClust HRM Software (Qiagen) in a supervised mode on the normalized melting curve.

CLCN1 mRNA analysis
Total RNA was extracted using the RNA mini Kit (Invitrogen) from muscle from an affected cat and a control random bred cat. Complementary DNA templates were synthesized using SuperScript III (Invitrogen, Carlsbad, CA) by reverse transcription of 1 μg of total RNA with gene specific primers (Table S1) and PolyT to obtain partial 5’ UTR, CDS and partial 3’ UTR. Each cDNA sample was subjected to PCR using primers (10 μM each) combined as follow: F1 - R1, F2 - R2, F3 - R3, F3 - R5, F4 - R4, F4 - R6, F5 - R5, F6 - R6, F7 - R7. The PCR conditions were: 1.5 mM Mg, 2 μl of cDNA in a total volume of 20 μl. The PCR cycle was conducted as previously described [57]. The PCR products with appropriate lengths were purified using the ExoSap (USB) enzyme per manufacturer’s recommendations. Purified genomic products were directly sequenced using internal primers (Table S1). Primers were designed in both UTRs and intronic regions, flanking the exons. Primers were tested for efficient product amplification on a DNA Engine Gradient Cycler (MJ Research, WA) with concentric (coaxial) needle electrodes while the cats were maintained under general gaseous anesthesia. Electromyography, nerve conduction velocities and repetitive nerve stimulations were performed within 10–15 minutes of induction of general anesthesia in three tested cats. Biopsies from the right biceps femoris muscle were collected under general anesthesia in three cats. The muscle biopsy was bisected: the first portion was placed in a saline-dampened gauze sponge followed by submission under refrigeration for histopathologic assessment (Comparative Neuromuscular Laboratory; University of California, San Diego). The second portion was transferred into RNA later (Qiagen, Valencia, CA). RNA was isolated using the Invitrogen RNA mini Kit (Invitrogen, Carlsbad, CA).

CLCN1 5’ss strength analysis
The strength of the 5’ splicing sites (5’ss) was calculated on nine bases fasta sequences (the last three bases of the exon and the first six bases of the adjacent intron) with the online program MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html). The program models short sequence motifs, such as those involved in RNA splicing, which simultaneously accounts for non-adjacent, as well as adjacent, dependencies between positions. This method is based on the ‘Maximum Entropy Principle’ and generalizes most previous probabilistic models of sequence motifs such as weight matrix models and inhomogeneous Markov models.

CLCN1 SNP genotyping
Genotyping was conducted using the High-Resolution Melting (HRM) assay. A real-time PCR was carried out on a Rotor-Gene Q Thermal cycler (Qiagen) and 239 samples from 28 populations were tested. PCR was performed in 10 μl reactions with 10 ng genomic DNA using the type-it HRM PCR kit (Qiagen) in accordance with the manufacturer’s instructions with HRM forward and reverse primers (Table S1). The assay was optimized using DNA from five wild-type samples and five affected samples, and DNA concentration was normalized to ~15 ng/μl for each sample to mimic average sample concentration from buccal swab isolation. Heterozygous samples for the assay optimization were made by combining wild-type and affected DNA with a 1:1 ratio. Graph of melting curves from the optimization are shown in Figure S2. A two-step cycling protocol consisting of an initial denaturation of 95°C for 5 min followed by 40 cycles of 95°C for 10 s and a combined annealing/extension step at 58°C for 30 s was carried out. The samples were subsequently subjected to a 65°C isotherm step for 90 s followed by a temperature gradient from 65°C to 85°C heating 0.1°C per step with 2 s wait at each step. Each genotype was analyzed in duplicate. The genotype was assigned using the Rotor-Gene ScreenClust HRM Software (Qiagen) in a supervised mode on the normalized melting curve.

Histopathology, immunohistochemistry and immunofluorescence
Unfixed specimens from the biceps femoris muscle of three cats affected with MC were shipped under refrigeration by an express service to the Comparative Neuromuscular Laboratory, University of California San Diego. Samples were immediately flash frozen in isopentane pre-cooled in liquid nitrogen then stored at −80°C until further processed. Control cat biceps femoris muscle was from the frozen tissue archives of the Comparative Neuromuscular Laboratory. Cryosections (8 μm) in myotonic cats and controls were cut and stained with H&E, or incubated with a mouse monoclonal antibody to myosin heavy chain slow (type 1 fibers, MyHC-slow, Leica Biosystems) or a mouse monoclonal antibody to...
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