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Clinical and molecular study of a new form of hereditary myotonia in Murrah water buffalo

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Abstract

Hereditary myotonia caused by mutations in *CLCN1* has been previously described in humans, goats, dogs, mice and horses. The goal of this study was to characterize the clinical, morphological and genetic features of hereditary myotonia in Murrah buffalo. Clinical and laboratory evaluations were performed on affected and normal animals. *CLCN1* cDNA and the relevant genomic region from normal and affected animals were sequenced. The affected animals exhibited muscle hypertrophy and stiffness. Myotonic discharges were observed during EMG, and dystrophic changes were not present in skeletal muscle biopsies; the last 43 nucleotides of exon-3 of the *CLCN1* mRNA were deleted. Cloning of the genomic fragment revealed that the exclusion of this exonic sequence was caused by aberrant splicing, which was associated with the presence of a synonymous SNP in exon-3 (c.396C>T). The mutant allele triggered the efficient use of an ectopic 5' splice donor site located at nucleotides 90–91 of exon-3. The predicted impact of this aberrant splicing event is the alteration of the *CLCN1* translational reading frame, which results in the incorporation of 24 unrelated amino acids followed by a premature stop codon.

Keywords: Hereditary myotonia; Chloride channel; Cryptic splicing; Water buffalo

1. Introduction

Myotonia is the phenomenon of delayed muscle relaxation after contraction [1]. Myotonia congenita is an inherited disorder of muscle membrane hyperexcitability caused by reduced sarcolemmal chloride conductance due to mutations in the skeletal muscle voltage-gated chloride channel gene (*CLCN1*), which encodes the main skeletal muscle chloride channel (CLC1) [2]. This disorder is the most common heritable skeletal muscle ion channelopathy in humans [3]. Nearly 30 years after the first description of myotonia in humans, a clinically similar disease was described in goats [4]. Studies performed on myotonic goats were initially important for delineating the physiological basis of myotonia and demonstrating the abnormal excitability of the muscle [5]. Subsequent studies

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in these animals revealed that there was a reduction in chloride conductance in the affected muscles [6–8]. A reduction in CLC1 conductance results in myotonia, a state characterized by muscle hyperexcitability [9]. Mutations in *CLCN1* that result in decreased activity of CLC1 [10] were first described in mice [11] and subsequently in humans [12,13], goats [14], dogs [15] and a horse [16].

Although there are clinical cases describing hereditary myotonia in calves [17] and cats [18–20], *CLCN1* mutations have not been identified in these species. Both autosomal recessive and dominant forms have been described in humans, whereas the disorder is inherited in an autosomal–dominant manner in goats [21]. The spontaneous mutations described in mice and the horse mutation are transmitted in an autosomal recessive manner [11,16,22].

In this study, we performed clinical, morphological and genetic evaluations of buffalo with a form of hereditary myotonia. This form of myotonia appears to be inherited in an autosomal recessive manner and is caused by an aberrant splicing process associated with the presence of a synonymous SNP. The result of this aberrant splicing event is the alteration of the *CLCN1* translation reading frame and the premature termination of translation.

2. Materials and methods

2.1. Clinical evaluation of affected animals

Eleven clinically affected Murrah buffalo of both sexes (7 females and 4 males) ranging in age from 1 day to 8 years obtained from herds in the states of São Paulo and Pará in Brazil were submitted to physical evaluation [23] and neurologic examination [24,25]. All protocols were approved by the Institutional Animal Care and Use Committee 231/11-CEUA.

2.2. Electromyographic evaluation

Electromyographical examination (EMG) with concentric needle electrodes at rest and during evoked effort was performed in standing position or lateral recumbency in 6 symptomatic buffalo with well-characterized clinical phenotypes and in 4 normal controls. The sensitivity was set to 20 μV/cm (at rest) or 200–1000 μV (during evoked effort), the analysis time to 10,200 or 500 ms/cm, and the filter band-pass to 20–10,000 Hz. A 2-channel Nicolet Compass Meridian, 2-channel ATI equipment or 2-channel Medelec Synergy Plinth were employed.

2.3. Muscle biopsies

Gluteus medius muscle biopsies were obtained from 4 phenotypically and electromyographically normal Murrah buffalo (control), 5 clinically affected buffalo and 2 clinically normal buffalo that were the parents of affected animals using the percutaneous needle biopsy technique as previously described [26]. The affected animals used in the study exhibited prominent action myotonia, generalized muscular hypertrophy and frequent bursts of repetitive discharges (myotonic runs) upon EMG. The initial biopsy site was identified by measuring 20 cm dorsocaudal to the tuber coxae of the ilium. The samples were removed at depth of 6 cm below the fascia, immediately frozen in liquid nitrogen and stored at −80 °C (for RNA extraction) or frozen by immersion in n-hexane precooled with liquid nitrogen and stored at −80 °C (for the histological and histochemical procedures).

2.4. Histological and histochemical analysis

The frozen muscle specimens were transferred to the cryostat chamber at −20 °C and fixed on metallic supports with Tissue-Tek OCT. Serial 8-μm sections were cut at −20 °C and processed for the histological and histochemical analysis using hematoxylin and eosin (H&E), the modified Gomori trichrome stain, the periodic acid-Schiff (PAS) stain, myosin ATPase after pre-incubation at pH 9.4 and 4.3, and nicotinamide-adenine-dinucleotide tetrazolium reductase (NADH-TR) [27].

2.5. RNA isolation

The frozen muscle samples (approximately 400 mg) were ground in a prechilled mortar and pestle, and total RNA was extracted using Trizol® (Invitrogen®, Gaithersburg, USA) following the manufacturer's instructions. The relative purity and quality of the isolated RNA was determined using a Nanodrop® 2000 Spectrophotometer (Thermo Scientific™, Wilmington, USA), and the A260-A280 ratio exceeded 1.8 for all preparations. The RNA samples were stored at −80 °C prior to analysis.

2.6. CLCN1 amplification and sequencing

Total RNA was treated with Deoxyribonuclease I Amplification Grade (Invitrogen®) to eliminate contaminating genomic DNA. Reverse transcription was conducted using the 3' RACE System (Rapid Amplification of cDNA Ends; Invitrogen®) following the manufacturer's instructions. The cDNA obtained was stored at −20 °C prior to amplification.

The complete *CLCN1* coding sequence was obtained using PfuTurbo® DNA Polymerase (Stratagene, La Jolla, USA) with combinations of primers 1–7 (Supplementary Table 1). The PCR products were analyzed via 1.5% agarose gel electrophoresis (Invitrogen®), stained with GelRed™ (Biotium™, Hayward, USA) and visualized under an ultraviolet light. The PCR products were directly purified with the QIAquick® PCR Purification Kit (Qiagen®, Valencia, USA); alternatively, if nonspecific products were observed, a product of the predicted size was excised from the gel and cloned into PCR II-TOPO using the Zero Blunt Topo® PCR Cloning Kit (Invitrogen®) following the manufacturer's instructions. Plasmid DNA was prepared