Mutational analysis of the equine Cu/Zn superoxide dismutase (SOD1) and amyotrophic lateral sclerosis (ALS2) in horses of Pakistani origin: No evidence found for motor neuron disease

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Abstract

Equine motor neuron disease (EMND) is a spontaneous neurologic disorder of adult horses which results from the degeneration of motor neurons in the spinal cord and brain stem. The etiology of EMND disease in the south Asian region is not well understood. To achieve the objective of the study, blood samples were collected from horses affected with EMND and DNA was purified by inorganic method. We sequenced both equine copper/zinc superoxide dismutase (SOD1) gene and equine ALS2 gene from 10 horses of Pakistani origin diagnosed with equine motor neuron disease (EMND) similar to amyotrophic lateral sclerosis (ALS) in humans. The 1359 nucleotides SOD1 coding region in the horse genome encodes 453 amino acids residues. Equine SOD1 exhibits 84 and 79.9% sequence identity to the human homolog at the nucleotides and amino acids levels, respectively. As a result, no mutation was found in SOD1 in any of the 10 affected horses while in case of horse ALS2 gene that consists of 41 exons, two missense (p.Val83Ile; p.Leu305Arg) and one synonymous (p.Thr410Thr) genetic variants were identified in the ALS2 gene. In addition, 50 normal control samples of Pakistani horses were sequenced representing the 40% of both the missense mutations. Our results suggest that both of the genes were not involved in causing motor neuron disease in horses.

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Introduction

Equine motor neuron diseases (EMND) are uncommon in domestic animals. Postmortem studies on afflicted horses show that weakness and muscle wasting results from degeneration of motor neurons in the spinal cord and brain stem (Cummings, 1990). Equine motor neuron disease (EMND) is a spontaneous neurologic disorder of adult horses. Clinical manifestations, pathological findings, and epidemiologic attributes resemble those of human motor neuron disease (MND) commonly known as Amyotrophic Lateral Sclerosis (Mohammed et al., 2007; Deng et al., 2010; Siddique et al., 2014; Xie et al., 2015). EMND differs from ALS; it does not involve alterations in any portion of the upper motor neuron (Turner et al., 2008). The horses afflicted with EMND lose 30% of the somatic motor neurons in the spinal cord and the brain stem before they manifest clinical signs (Polack et al., 1998; Mohammad et al., 2012). In the cytoplasm, SOD1 dismutates superoxide anion radical to $H_2O_2$ that is further reduced to $H_2O$ by catalase, glutathione peroxidases or peroxiredoxins (Rhee et al., 2005). In intermembrane space (IMS), SOD1 has been suggested to play the similar protective role in handling of superoxide as in the cytosol (Lindsey et al., 2011). However, in this location, the scavenging systems might not be efficient enough to eliminate the $H_2O_2$ produced by dismutation. Upon cellular stress and pathological conditions such as ALS, the elevated $H_2O_2$ levels could contribute to mitochondrial damage (Vehtilaïinen et al., 2014). A genetic linkage has been demonstrated between the inherited form of amyotrophic lateral sclerosis (ALS) and the SOD1 locus, specifically involving point mutations in this gene (Pramatarova et al., 1995). Almost 90% of ALS cases occur sporadically while 20% of familial amyotrophic lateral sclerosis (FALS) has been reported due to mutations in Cu/Zn superoxide dismutase (SOD1), a major cytosolic antioxidant enzyme in eukaryotic cells (Green et al., 2002). As in ALS, the etiology of this neurologic condition of horses is still unresolved. Equine SOD1 was considered as a candidate gene to elucidate the etiology of EMND. Evidence of peroxidative injury, lower SOD1 activities, decreased plasma levels of vitamin E in EMND cases relative to controls and the clinical pathologic similarities between EMND and ALS suggest that oxidative stress in the motor neurons also contributes to the pathogenesis of EMND (Divers et al., 1997).

Horses which are deprived of pasture or green, high-quality hay, and which are not supplemented with vitamin E for more than a year, are at greatest risk for EMND (Mohammed et al., 2007) and has been reported to be associated with some cases of ALS (Ascherio et al., 2005) which suggests an increased oxidative stress may be involved in development of these diseases. The horse ALS2 in horse genome is present on chromosome 18 while on the other hand in human genome ALS2 gene is located on chromosome 2q33, composed of 34 exons (Kress et al., 2005). The horse SOD1 gene in horse genome is present on chromosome 26 while in human genome, the SOD1 gene is located on chromosome 21q22.1, spans about 9.3 kb of genomic DNA and encodes for a 153-amino acid long protein of 16 kDa (Gros-Louis et al., 2006). The SOD1 coding region, organized into five exons. SOD1 gene has been sequenced in several higher eukaryotes including the cow, mouse, rat, rabbit and humans.

During this study, we amplified and sequenced the equine SOD1 and ALS2 genes by PCR in a total of 10 affected horses. The objective of the study was to determine whether SOD1 and ALS2 genes mutations are linked to EMND or not. Our results showed that both of the genes were not involved in causing motor neuron disease in horses.

Materials and methods

Collection of blood samples and clinical analysis

A total of 10 horses diagnosed with EMND were enrolled based on the history of disease, clinical signs, complete physical examinations such as weight loss, evidence of muscle atrophy, weakness, short strides, trembling, head hanging and muscle fasciculation. A total of 5 ml blood samples from jugular vein of the horses were collected and processed for DNA extraction (Grimberg et al., 1989).
Primer designing, DNA isolation and exons amplification

Primers for exons amplification and subsequent sequencing of genes SOD1 and ALS2 were designed by using online software the primer3 web site (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) to flank all exon–intron boundaries. Based on the nucleotide sequence of the horse SOD1 and ALS2, we synthesized five sets of PCR oligonucleotide primers for SOD1 and 34 sets of primers for 41 ALS2 exons of horse genome.

Genomic DNA was isolated by using simple inorganic method (Grimberg et al., 1989). Genomic DNA of horses was used as a template in the PCR reaction. PCR reactions contained 50 ng/µl of genomic DNA in a total volume of 20µl PCR reaction.

Reactions were performed in 0.2 ml PCR tubes on a Bio-Rad™ thermo-cycler. Amplifications of exons was performed with an initial activation step at 93°C for 3 min following by first 10 cycles as touchdown PCR (with annealing temperature from 64°C to 54°C or 67°C to 57°C) and additional 20 cycles with denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 45 sec with a final extension at 72°C for 7 minutes. Amplified products were run on 2% agarose gel in TBE buffer at 110 V for 45 minutes and were visualized by staining with ethidium bromide under UV trans-illuminator.

DNA Sequencing

For DNA sequencing of SOD1 gene, genomic DNA was amplified by PCR using exons specific primers, later these PCR products were used as a template for DNA sequencing. Sequencing reaction were prepared by using 15µl from the above prepared EXO-SAP solution; the following reagents were added in specific amounts as, 6µl of diluted PCR product, 2µl of big dye sequencing mix, 1µl of primer (3.2µM), and 1µl of 5X dilution buffer. PCR were carried using thermocycler program of 30 basic cycle of standard PCR. After exons amplification, all exons and exon/intron border regions were sequenced and analyzed by Bio Edit (version 7.0.2) for mutation analysis. Reference sequences were used from UCSC Genome Browser.

Results

A total of 10 horses diagnosed with EMND were analyzed based on the history of disease, clinical signs, and complete physical examinations such as weight loss, evidence of muscle atrophy, weakness, short strides, trembling, head hanging and muscle fasciculation. The deep analysis of affected horses concluded that all the horses were affected with motor neuron disease subsequently equine SOD1 gene and ALS2 were sequenced.

**Fig. 1.** Genetic Variant (c.247G>A) in exon 3 of horse ALS2 gene showing a missense substitution (p.Val83Ile).
During current study, no mutation was found in gene SOD1 concluding that motor neuron disease of horses is not linked to mutations of SOD1. While on the other hand, ALS2 gene consists of 41 exons and all exons are coding in horses. During current study, two missense (p.Val83Ile; p.Leu305Arg) and one synonymous genetic variant (p.Thr410Thr) were identified in exon 3 and 4 of ALS2 gene of the affected horses.

**Fig. 2.** Genetic Variant (c.914T >G) in exon 3 of horse ALS2 gene showing a missense substitution (p.Leu305Arg).

**Discussion**

During current study, mutational analysis was performed of gene SOD1and ALS2 on the horses affected with motor neuron disease. Gene SOD1 consists of five exons and all are encoding (Saccon et al., 2013) both, in humans and horses. In humans, SOD1 is present on chromosome 21q22 while in horses, it is present on chromosome 26. The human SOD1 spans 11 kb on chromosome 21and encodes a homodimeric enzyme of approximately 32kDa and 153 amino acids per subunit (Gros-Louis et al., 2006; Diez et al., 2016). During current study, mutational analysis was performed of gene SOD1 on the horses affected with motor neuron disease. All the affected horses having signs and symptoms of MNDs; DNA sequencing of all the exons did not show any mutation in horse gene SOD1. Earlier in 1994, in another study performed by (Rua-Domenech et al., 1994) on equine motor neuron disease on 16 affected horses, no mutation was identified in gene SOD1. The ALS2 gene belongs to a family of genes called ARHGEF (Rho guanine nucleotide exchange factors). ALS2 gene encodes for a protein called ALS2 or alsin. Alsin is a GEF protein predominantly expressed in central nervous system (Sillevis-Smitt and De Jong, 1988). Alsin is produced in wide range of tissues especially large amount in brain and particularly in motor neurons (nerve cells in the brain and spinal cord that control the movement of muscle). Human ALS2 gene (OMIM, 606352) consists of 34 exons, while horse ALS2 gene consists of 41 exons. During current study, mutational analysis of gene ALS2 was performed on the horses affected with motor neuron disease. Sequence analysis of ALS2 gene in horses identified two missense and one synonymous mutation in exon 3 and exon 4 of ALS2 gene in the affected horses originating from Pakistan. A total of 50 normal control samples of Pakistani horses were sequenced representing the 40% of both the missense mutations. Our results suggest that both of the genes were not involved in causing motor neuron disease in horses of Pakistani origin.
Fig. 3. Genetic Variant (c.1230G >A) in exon 4 of horse ALS2 gene showing a synonymous change (p.Thr410Thr).

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