INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 3, p. 157-162, 2017

RESEARCH PAPER OPEN ACCESS

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

Shakeela Daud[1,2], Sara Naudhani[3], NisarAhmed[2], Tahir Yaqub[4], Abu Saeed Hashmi[1], Ali Raza Awan[1], Abdul Wali[2], Muhammad Luqman[*3], Muhammad Wasim[1]

[1]*Institute of Biochemistry and Biotechnology (IBBt), UVAS, Lahore, Pakistan*

[2]*Department of Biotechnology, BUITEMS, Quetta, Pakistan*

[3]*Department of Environmental Science, BUITEMS, Quetta, Pakistan*

[4]*Department of Microbiology, UVAS, Lahore, Pakistan*

Key words: EMND-*SOD1*, Equine *ALS2*, Mutation, Horse.

http://dx.doi.org/10.12692/ijb/10.3.157-162 Article published on March 17, 2017

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.

* **Corresponding Author:** Muhammad Luqman ✉ hyphomycetes@yahoo.com

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_2produced by dismutation. Upon cellular stress and pathological conditions such as ALS, the elevated H_2O_2 levels could contribute to mitochondrial damage (Vehvilainen *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.

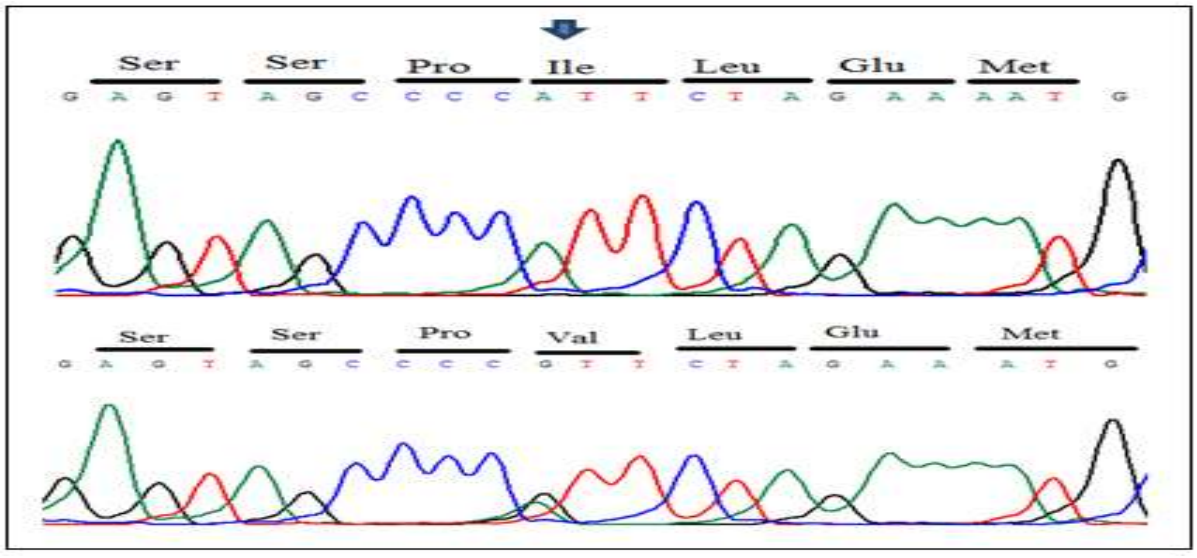

Fig. 1. Genetic Variant (c.247G>A) in exon 3 of horse *ALS2* gene showing a missense substitution (p.Val83Ile).

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.

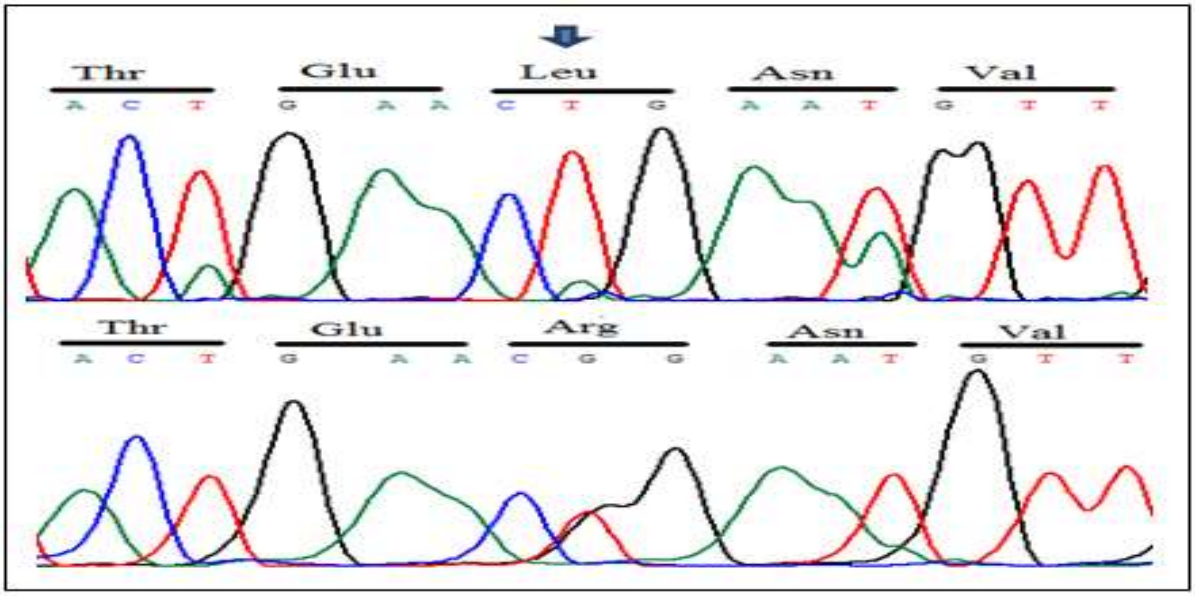

Fig. 2. Genetic Variant (c.914T >G) in exon 3 of horse *ALS2* gene showing a missense substitution (p.Leu305Arg).

Discussion

During current study, mutational analysis was performed of gene *SOD1*and *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 3and 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.

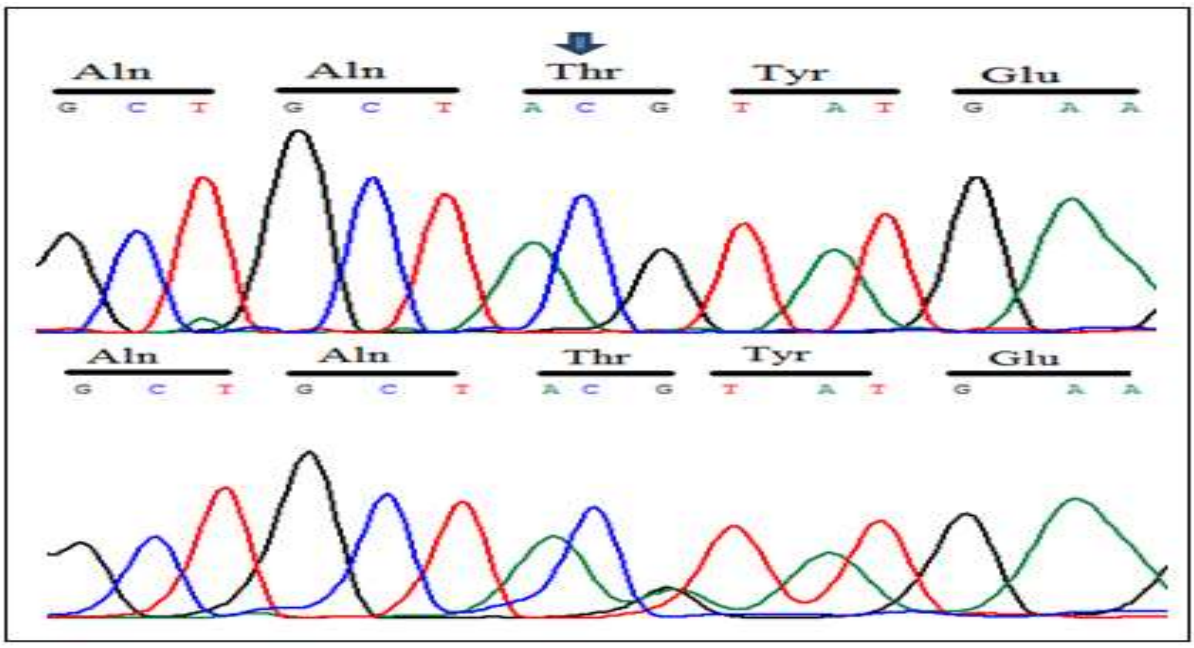

Fig. 3. Genetic Variant (c.1230G >A) in exon 4 of horse *ALS2* gene showing a synonymous change (p.Thr410Thr).

Acknowledgment

We are thankful to Dr. Azeem and Muhammad Akram for their cooperation and providing blood samples of affected horses from race club Lahore. We acknowledge the financial support for current research work by ORIC, BUITEMS, Quetta.

References

Ascherio A, Weisskopf MG, O'reilly EJ, Jacobs EJ, McCullough ML, Calle EE, Cudkowicz M, Thun MJ. 2005. Vitamin E intake and risk of amyotrophic lateral sclerosis. Annals Neurology **57(1),** 104-10.

Cummings JF, George C, de Lahunta A, Fuher L, Valentine BA, Cooper BJ. 1990. Equine motor neuron disease. A preliminary report. Cornell Veterinarian **80,** 357-379.

Deng HX, Zhai H, Bigio EH, Yan J, Fecto F, Ajroud K, Mishra M, Ajroud-Driss S, Heller S, Sufit R, Siddique N, Mugnaini E, Siddique T. 2010. FUS-immunoreactive inclusions are a common feature in sporadic and non-*SOD1* familial amyotrophic lateral sclerosis. Annals Neurology **67(6),** 739-748.

Diez DCE, Zafra R, Acevedo LM, Perez J, Acosta I, Rivero JLL, Aguilera-Tejero E. 2016. Eosinophilic Enteritis in Horses with Motor Neuron Disease. Journal of Veterinary Internal Medicine **30(3),** 873–879.

Divers TJ, Mohammed HO, Cummings JF. 1997. Equine motor neuron disease. Veterinary Clinics of North America: Equine Practice **13,** 97–105.

Lindsey RF, Anissa I, Jordi M, Yingjie L, Jason MH, Giovanni M, Jonathan DG. 2011.*SOD1* targeted to the mitochondrial intermembrane space prevents motor neuropathy in the *SOD1* knockout mouse. Brain **134(Pt1),** 196-209.

Green, SL, Tolwani RJ, Varma S, Quignon P, Galibert F, Cork LC. 2002. Structure, chromosomal location, and analysis of the canine Cu/Zn superoxide dismutase (*SOD1*) gene. Journal of Heredity **93(2),** 119-124.

Grimberg J, Nawoschik S, Belluscio L, McKee R, Turck A, Eisenberg AA. 1989. Simple and efficient non-organic procedure for the isolation of genomic DNA from blood. Nucleic Acids Research **17,** 83-90.

Gros-Louis F, Gaspar C, Rouleau G. 2006. Genetics of familial and sporadic amyotrophic lateral sclerosis. Biochimica et Biophysica Acta **1762(11-12),** 956-972.

Kress JA, Kühnlein P, Winter P, Ludolph AC, Kassubek J, Müller U, Sperfeld AD. 2005. Novel mutation in the *ALS2* gene in juvenile amyotrophic lateral sclerosis. Annals Neurology **58(5),** 800-803.

Mohammed HO, Divers TJ, Summers BA, de Lahunta A. 2007. Vitamin E deficiency and risk of equine motor neuron disease. Acta Veterinaria Scandinavica **49(1),** 17.

Mohammed HO, Divers TJ, Kwak J, Omar AH, White ME, de Lahunta A. 2012. Association of oxidative stress with motor neuron disease in horses. American Journal of Vetrinary Research **73(12),** 1957-62.

Polack EW, Cummings JF, King J, Mohammed HO. 1998. Morphometric studies on equine motor neuron disease. Equine Veterinary Journal **30,** 255-259.

Pramatarova A, Figlewicz DA, Krizus A, Han FY, Ceballos- Picot I, Nicole A, Dib M, Meininger V, Brown RH, Rouleau GA. 1995. Identification of new mutations in the Cu/Zn superoxide dismutase gene of patients with familial amyotrophic lateral sclerosis. American Journal of Human Genetics **56,** 592- 596.

Rhee SG, Yang KS, Kang SW, Woo HA, Chang TS. 2005. Controlled elimination of intracellular H(2)O(2), regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. Antioxidants and Redox Signaling, **7(5-6),** 619-26.

Rua-domenech DI, Wiedmann RM, Batt CA, Mohammed HO, Cummings JF, Divers TJ. 1994. Equine motor neuron disease: molecular epidemiologic studies. The kenya veterinarian **18(2),** 290-292.

Saccon RA, Bunton-Stasyshyn RK, Elizabeth MC, Fratta P. 2013. Is *SOD1* loss of function involved in amyotrophic lateral sclerosis? Brain **136(Pt 8),** 2342-2358.

Siddiqi S, Foo JN, Vu A, Azim S, Silver DL, Mansoor A, Tay SK, Abbasi S, Hashmi AH, Janjua J, Khalid S, Tai ES, Yeo GW, Khor CC. 2014. A novel splice-site mutation in ALS2establishes the diagnosis of juvenile amyotrophic lateral sclerosis in a family with early onset anarthria and generalized dystonias. PLoS One **9(12),** e113258.

Sillevis-Smitt PAE, De Jong JMBV. 1988. Animal models of amyotrophic lateral sclerosis and the spinal muscular atrophies. Jounal of Neurological Sciences **91,** 231- 258.

Turner BJ, Baumer D, Parkinson NJ, Scaber J, Ansorge O, Talbot K. 2008. TDP-43 expression in mouse models of amyotrophic lateral sclerosis and spinal muscular atrophy. BMC Neuroscience **9,** 104

Vehvilainen P, Koistinaho J, Gundars G. 2014. Mechanisms of mutant *SOD1* induced mitochondrial toxicity in amyotrophic lateral sclerosis. Frontier in Cellular Neuroscience **8,** 126.

Xie F, Cen ZD, Xiao JF, Luo W. 2015. Novel compound heterozygous ALS2 mutations in two Chinese siblings with infantile ascending hereditary spastic paralysis. Neurological Sciences **36(7),** 1279-80.