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Original scientific article

POLYMORPHIC STATUS AND PHYLOGENETIC ANALYSIS OF MYOSTATIN GENE IN PAK-THOROUGHBRED

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Raza Z. H., A. Nadeem, M. Javed, F.-ul-Hassan, W. Shehzad, A. Ahmad, M. Saadullah, M.M.Ali, (2020). *Polymorphic status and phylogenetic analysis of myostatin gene in Pak-thoroughbred*.- Genetika, Vol 52, No3, 1281-1290.

Myostatin is a protein translated by the the *MSTN* gene (also known as GDF8), is responsible for limiting muscle growth and strength. In thoroughbred horse (*Equus Caballus*), limited studies have been designed to examine the variants in the coding region of *MSTN* gene. However, no data is available regarding the single nucleotide polymorphisms (SNPs) of the *MSTN* about racing performance in thoroughbred horses of Pakistan. In this study blood samples of fifteen Pakistani thoroughbred horses were collected from Race Club Lahore and immediately transferred into the ice box. The DNA was extracted by using phenol-chloroform method. Primers were designed for the

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amplification of all exons of the *MSTN* gene. The amplified PCR products were precipitated and sequenced for the identification of SNPs. SNPs were identified by visualizing the peaks of sequenced data by using Chromas Software. Phylogenetic analysis of MSTN gene in Pak-thoroughbred with racing species and some breeds of horses like Marwari Indian breed, Sindhi breed, Kathlawari breed, Italian breed and Chines breed was done separately by using MEGA 6 software. The analysis of identified SNPs were carried out by software SNPator. The sequenced data with altered protein was published in GenBank with accession number MN604194. Results have shown a total of 3 single nucleotide polymorphisms through Blast with reference sequences. Two SNPs were found in exon 2 at position of 2406 (C/T) and 2408 (C/T) respectively. One SNP (T/C) was detected in exon 3 at the position of 4661. In conclusion, Pak-thoroughbred horse population has 3 polymorphisms in their coding region which can be used as a biological marker for athletic abilities in Pak-thoroughbred.

*Keywords: m*yostatin, Pak-thoroughbred, polymorphism, horses, phylogenetic analysis

INTRODUCTION

In horses, metabolic pathways, types of muscle fibers, muscle proliferation and muscle contraction respond to exercise, training and it is also genetically influenced. These traits can determine the performance of an elite athletic horse (RIVERO & HILL, 2016). Studies confirm the genetic control is responsible for influencing the athletic performance of Thoroughbred horses (MOON *et al*., 2015). Besides the epigenetic factor, it is accepted that athletic performance capabilities are also influenced by genetic factors (GAFFNEY and CUNNINGHAM, 1988).

The retention of the *MSTN* allele is due to selective breeding. Myostatin protein (GDF-8) inhibits myogenesis (muscle growth and differentiation), translated by the *MSTN* (JIN & BOJIANG, 2018; MCPHERRON *et al.*, 1997). Myostatin gene is the best candidate while studying related muscle growth (GRADE *et al.*, 2019). MSTN gene polymorphism is associated with muscle enhancement (MENG, WANG, QIU, LIU, & WANG, 2017; WADOOD *et al*., 2019; ZHANG et al., 2019).The *MSTN* variants have been associated with differential athletic capability in horses (AHAD *et al.*, 2017; HILL *et al.*, 2010). One of the intronic SNP in the equine *MSTN* is important to predict optimal racing distance (BOWER *et al*., 2012). Thus *MSTN* locus variation has an impact on muscle formation during early development and optimizes athletic capability (BOWER *et al*., 2012). Thoroughbred horses for best short, middle distance races and greater stamina rely on homozygous C/C horses, heterozygous C/T, *and* homozygous T/T horses, correspondingly (HILL *et al*., 2010).

Studies elucidate that *MSTN* has a pleiotropic as well as rate-limiting effect on muscle formation. There is a relationship between the *MSTN* with muscle hypertrophy (BOULEY *et al*., 2005).

However, no documented evidence has been found about the genotyping by sequencing of *MSTN* in thoroughbred horses of Pakistan. Therefore, the current study was aimed to identify SNPs of *MSTN*, the diversity among thoroughbred horses of Pakistan and their phylogenetic analysis based on the *MSTN* sequence.

MATERIAL AND METHODS

Animal Selection and Samples Collection

A total of 15 horses were selected from Lahore Race Club. These horses were comprised of normal healthy horses with wining capability in 800-1600 meter races (Intermediate Racing Distance). Horses from both sexes, aged > 2 years were selected. Whole blood was collected from each individual and stored in vacutainer containing 0.25% ethylenediaminetetraacetic acid (EDTA). Blood samples were stored in ice after sampling and then transferred temporarily to the 4C before DNA extraction.

DNA Extraction, Primer Designing and Amplification

DNA isolation was carried out by using the phenol-chloroform method (THOMAS, MORENO, AND TILZER, 1989). Extracted DNA was eluted by Polymerase Chain Reaction graded water. Storage conditions for DNA were set at -20º C to prevent the degradation. DNA was quantified by both qualitative (Gel electrophoresis) and quantitative methods (Nanodrop 2000, Thermo Scientific USA). Three sets of primers (Table 1) were designed by using Primer3 software (www.primer3.com) for all three exons of *MSTN* from the reference sequence (Accession no. AY840554) (WANG *et al.*, 2006).

Table 1. Three pairs of primers were designed for the coding region of the MSTN gene

S.No.	Primers ID	Sequence	GC %	Tm	Length	Product Size
1	MSTNE1_F	GTGGAGCAGGAGCCAATCAT	55	60.11	20	705
	MSTNE1_R	TGCTTACATACAAGCCAACAGC	45.45	59.51	22	
2	MSTNE2_F	TGTTCACCAACTAATATGGAAGGGT	40	59.99	25	593
	MSTNE2_R	GCCATTGGGGTAAGATGCCT	55	60.11	20	
3	MSTNE3_F	TGAGGTAGGAAAGTGTTTCGGG	50	59.96	22	502
	MSTNE3_R	TCAAAATTGTGAGGGGAAGGC	47.62	58.75	21	

The exonic portion was amplified by designed primers. PCR conditions are mentioned in Table 1. The confirmation of PCR amplification was done by visualizing the bands on agarose gel in the gel documentation system (Bio-Rad, USA).

Sequencing and Bioinformatics Analysis

The PCR product was genotyped by the chain-termination method. These sequences were published in Gen-Bank with the Accession number of MN604194. Alignment of sequences was carried out using nucleotide blast (https://blast.ncbi.nlm.nih.gov) (TATUSOVA and MADDEN, 1999). SNPs were identified by visualizing the peaks of sequenced data by using Chromas Software (2.5). The phylogenetic analysis of the *MSTN* of Pak thoroughbred was carried out in two ways, firstly with the different breeds of horses including Marwari breed, Sindhi breed, Italian horse, Anglo Arabian breed and Chines breed; secondly with the species that have the tendency of racing such as camels, tiger, racing bull, wild cat, lion and donkeys. The analysis

was done by using MEGA 4.1 software. The Maximum Composite Likelihood method was used to identify the closest relation.

RESULTS AND DISCUSSION

Identification of Single Nucleotide Polymorphism

A total of three SNPs was detected through blast with the reference sequences. The first two SNPs were found in exon 2 at the position of 2406 (C/T) and 2408 (C/T). The third SNP (T/C) was detected in exon 3 at a position of 4661.

All three polymorphisms were observed in the coding region of the *MSTN* in thoroughbred horses of Pakistan. Though there is only one amino acid change in the polypeptide chain of the *MSTN* protein as Valine (Val) is replaced by Methionine (Met).

Phylogenetic Analysis

The phylogenetic analysis with different racing species showed that the MSTN in Pak thoroughbred has an independent clad. *Panthera pardus, Panthera tigris, Camelus dromedaries and Felis catus* were found the most closely related to Pak-Thoroughbred (Figure 1). Out clad was found which indicates that the sequence of MSTN gene with respect to other species has a little difference. In present study the results also supported this phylogenetic tree because in Pak Thoroughbreds a significant change in sequence is found as single nucleotide polymorphisms. Although, the previous studies have shown that the *MSTN* is highly conserved (MCPHERRON *et al*., 1997) but slight changes in the sequences can be observed in different species.

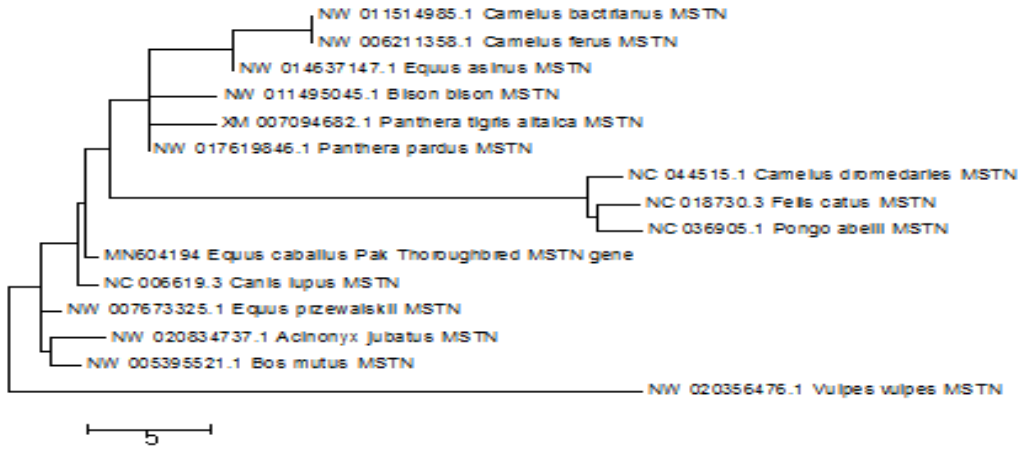

Figure 1. Phylogenetic analysis of MSTN gene of Pak Thoroughbred with Racing Species

The phylogenetic analysis within the breeds of horses revealed that the Pak-thoroughbred MSTN gene is closely related to Chines Breeds. It is revealed that gene variants are associated with geographical regions (HOSHIDE *et al*., 2014) because China shared the border with Pakistan. Arabian and Italian breed of horse fall in distant clad which also indicate that similarities of gene sequences are associated with geographical regions.

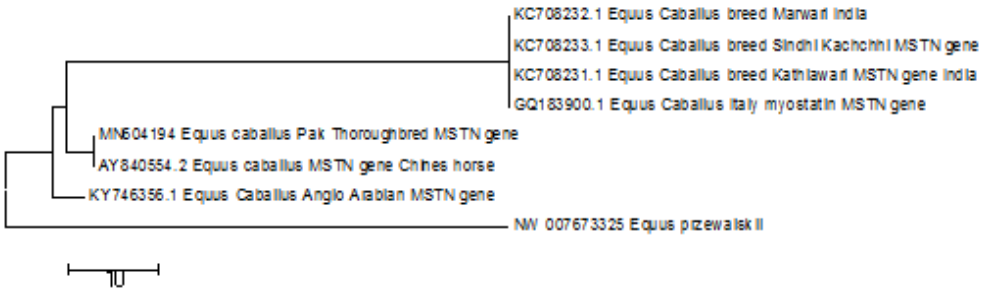

Fig. 2 Phylogenetic analysis of MSTN gene of Pak thoroughbred with different horse breeds

Altered Amino Acid

SNPS in MSTN gene of thoroughbred horses of Pakistan caused the amino acid change in the polypeptide chain. Observed polymorphisms in polypeptide chain changed Valine (Val) into Methionine which alters the protein. M/V change can change the protein function (HE *et al.*, 2013). Methionine and valine are amphipathic and hydrophobic respectively. Change in one amino acid-like M/V can change the protein structure and it can be predicted too (SCHAEFER and ROST, 2012). The variations in the *MSTN* gene lead to the premature stop codon and ultimately altered protein which cause the extra-ordinary muscle development and powers in children (SCHUELKE *et al.*, 2004).

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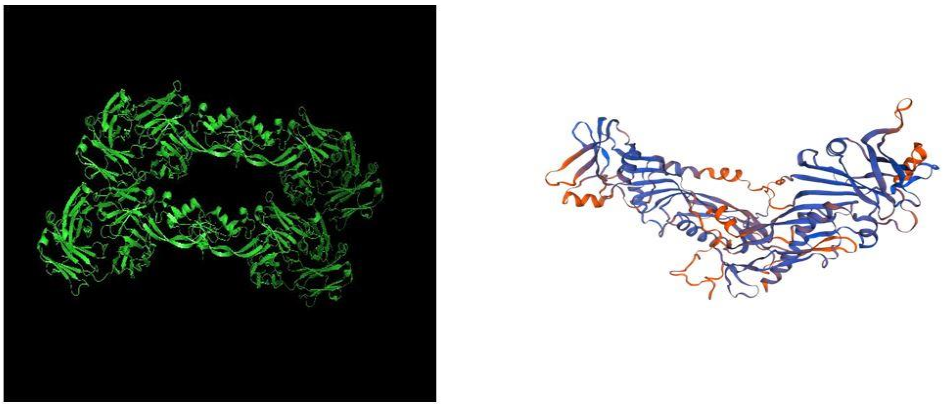
Query  1    MQKLQIYVYIYLFMLILAGPVDLNENSEQKENVEKEGLCNACTWRQNTKSSRIEAIKIQI  60
            MQKLQIYVYIYLF+LILAGPVDLNENSEQKENVEKEGLCNACTWRQNTKSSRIEAIKIQI
Sbjct  1    MQKLQIYVYIYLFVLILAGPVDLNENSEQKENVEKEGLCNACTWRQNTKSSRIEAIKIQI  60
```

Figure 3. Change in amino acid sequence due to SNPs found in Pak thoroughbreds



Original Protein (with Valine) *Altered protein (with Methionine)*

Figure 4. Protein structure with wild type allele of MSTN gene and altered allele

Polymorphism of the *MSTN* affects the traits (HAN *et al*., 2012). The Myostatin gene plays a vital role in muscle mass formation (MENDIAS *et al*., 2015). Single nucleotide polymorphism can be used as an effective bio-marker for appearance of traits (GUANG-XIN, YONG-FU, JIAN-NING, WEI-WEI, and YONG-JU, 2016). One of the intronic SNP in the equine the *MSTN* is important to predict optimal racing distance (BOWER *et al*., 2012). A SNP in the non-coding region of MSTN gene is associated with the athletic performance of the elite horse (VAN DEN HOVEN *et al*., 2015). Genotyping revealed that Paint and Quarter horses are associated with different muscle fibers and increased sprint (PETERSEN, MICKELSON, CLEARY, and MCCUE, 2013). Two SNPs in the promoter sequence at 646 bp upstream g.26T> C and 516bp upstream g.156T> C to start codon and Five SNPs were identified in the intronic sequences,4 in intron 1 and 1 in intron 2 which has an association with racing (DALL'OLIO *et al*., 2010). The present study is different from previous because in previous studies promoter and intronic regions were more focused for the identification of SNPs. On the other hand, in current study coding region was targeted. MSTN gene variation is linked with the athletic capability of TBHs (BINNS, BOEHLER, and LAMBERT, 2010). SNPs in MSTN gene are linked with the change of morphological peculiarities (DALL'OLIO *et al*., 2010). A total of 3 mutations were documented in Icelandic horses which had the association with physical strength (FRANÇOIS *et al*., 2016). A total of 189 TBHs that won races in North America were genotyped and there were 2 SNPs observed in the myostatin gene ($p<0.001$), revealed after association test (BINNS *et al*., 2010). MSTN gene polymorphism, itself affected the racing performance of Thoroughbred racehorses, and racing performance is heritable (TOZAKI *et al*., 2010). A study elucidated that 6 SNPs were noticed in the 1st intron of MSTN gene, had a strong association for a race; stamina and sprinting. The C/C horse's suits shot distance, and fast races, horses with C / T favor medium-distance racing and horses with T / T had the great stamina than other (HILL *et al*., 2010). In the present study, it was identified that Pak thoroughbreds have C/T gene variant in 3rd exon which indicates that these horses have a good potential for medium-distance racing. On chromosome 18, a total of 4 SNPs, T>C, G>T, C>T, and A>G, were identified in 91 thoroughbred horses in training to find an association of genotype with morphology, which includes body weight, chest circumference, withers height, body weight/withers height and cannon circumference (TERUAKI TOZAKI *et al*., 2011). In the present study, 2 Polymorphisms T/C in exon 2nd were identified that confirms that agree the results of previous study of TOZAKI *et al*. in which it was confirmed that T/C allele has involved in morphology of muscles in order to enhance the muscle strength. Untrained horses with C/C allele, exhibited the immense high level of MSTN's mRNA and showed a prominent decrease for mRNA, equal to −5.88 fold after training, comparing C/ T least and T/ T TBHs intermediate decrease after exercise (BOWER *et al*., 2012). Therefore, by comparing these results, it can be inferred that C/T variant identified in current study involved in lower down the mRNA exhibition which ultimately improve the muscle fiber building capabilities. SNP for racing ability was situated in the MSTN region (MCCUE *et al*., 2012). This study also a strong confirmation of the reported results. The physiological performance parameters were assessed

for 85/102 TBHs of a training yard, which were being trained for Flat racing. Genotypes at g.66493737-C/T determine variations in speed. All TBHs performed well, amongst them C/ C TBHs had greater speed than T/ T TBHs, measurements for C/T individuals were in between to homozygote horses (MCGIVNEY *et al*., 2012). In the present study the identified C/T variant indicate that Pak thoroughbreds are suitable for the medium-distance racing. A total of six SNPs comprising two new SNPs, g.587A > G, and g.598C > T, comparative to previous studies were recognized in 15 Chinese horse breeds which was associated with racing performances (LI *et al*., 2014). Allele C of g.66493737-C>T SNP, had a relationship with racing in TBHs, was substantially fixed in Quarter Horses and confirms association with racing and heavy body-weight (PEREIRA *et al* 2016). These studies also confirm the relation of C/T allele identified in Pak thoroughbreds with racing performance. Two SNPs, g.66495826-T>C and g.66495696-T>C explicated 3 haplotypes TT, TC and CT with frequencies 0.877, 0.101, and 0.005, respectively were identified a distinct haplotype CC with a frequency value of 0.016 was found in Polish Heavy Draft after investigating promoter MSTN in 451 horses belonging to 5 Arabian, Polish, Thoroughbred, Hucul, Polish Heavy Draft and Konik breeds (STEFANIUK *et al*., 2016). The g.66493737-T/C was suited markers for evaluation of the ability of non-elite TBHs for racing at short/long distances (VAN DEN HOVEN *et al*., 2015). These studies have showed that of T/C gene variants observed in the present study can be used as a biological marker for racing abilities. It is observed that SNPs in racing genes of horses can control muscles formation besides racing ability (STEFANIUK *et al*., 2016).

It is concluded that Pak-thoroughbred horse population has three polymorphisms in their coding region of the MSTN gene: two in 2nd exon and one in 3rd exon. However, there was no SNP identified in the 1st exon. Moreover, it is observed that these SNPs altered the peptide chain. Further proteomic based studies are recommended for the identification of the nature of the altered protein. Other non-elite Pakistani thoroughbred horses and local breeds should also be studied for the confirmation of such SNPs.

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POLIMORFNI STATUS I FILOGENA ANALIZA MYOSTATIN GENA KOD PAK-PUNOKRVNIH

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Izvod

Miostatin, Na punokrvnom konju (Ekuus Caballus), ograničene studije su osmišljene da ispitaju varijante u kodnom regionu MSTN gena. Međutim, nisu dostupni podaci o SNP MSTN o trkačkim performansama kod rasnih konja Pakistana. U ovoj studiji uzorci krvi petnaest pakistanskih punokrvnih konja prikupljeni su iz Race Club Lahore. DNK je ekstrahovana metodom fenol-hloroform. Prajmeri su dizajnirani za amplifikaciju sva tri egzona gena MSTN. Pojačani PCR proizvodi su istaloženi i sekvencirani za identifikaciju SNP-a. SNP-ovi su identifikovani vizualizacijom pikova sekvenciranih podataka pomoću softvera Chromas. Filogenetska analiza gena MSTN u Pak punokrvnom uzgoju sa raznim trkačkim vrstama i nekim vrstama konja poput indijske pasmine Marvari, rase sindhi, rase Kathlavari, italijanske rase i rase Chines rađena je odvojeno pomoću softvera MEGA 6. Analiza identifikovanih SNP-a izvršena je pomoću softvera SNPator. Podaci sa izmenjenim proteinima objavljeni su u GenBank-u sa pristupnim brojem MN604194. Rezultati su pokazali ukupno 3 polimorfizma pojedinačnih nukleotida kroz Blast sa referentnim sekvencama. Prva dva SNP-a pronađena su u eksonu 2 na položaju 2406 (C / T), odnosno 2408 (C / T). Treći SNP (T / C) otkriven je u eksonu 3 na položaju 4661. U zaključku, pakistanska čistokrvna populacija konja ima 3 polimorfizma u svom kodnom području, koji se mogu koristiti kao biološki markeri za sportske sposobnosti kod čistokrvnih pasmina.

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