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Original Article

Genetic Association of MSTN Gene Variant (18:66493737T>C) with Track Performance & Muscle Development in Pakistani Horses

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ABSTRACT

The horse, revered for its diverse traits including racing prowess, gaitedness, and distinctive behavior, plays a pivotal role in various sports. Numerous studies have linked racing performance in horses to the MSTN gene across global populations. **Objectives:** To investigate the genetic variability of the 18:66493737T>C variant in Pakistani random-bred horses. **Methods:** ARMS-PCR was employed where 24 horses sourced from UVAS equine clinic were genotyped. **Results:** Revealing a population distribution of 54% homozygous wild-type (TT), 41% heterozygous (TC), and 4% homozygous mutant (CC) at this locus. The alternative allele frequency within elite performers and control horses stood at 0.36 and 0.12, respectively. Application of the Chi-Square association test using the PLINK data toolset yielded a highly significant p-value of 7.832×10^{-6}. **Conclusions:** This underscores significant genetic variability at the locus in the Pakistani horse population, aligning with global patterns. Future studies are advocated, incorporating racing performance data and encompassing diverse indigenous horse breeds with substantial sample sizes. Identification of subject markers can inform targeted breeding strategies, contributing to the enhancement and preservation of desirable traits across various horse breeds.

INTRODUCTION

Horse (*Equus caballus*) racing is one of the ancient sports that has gained popularity with time in the modern era [1]. It has developed as a public recreation to utilize their leisure time. Moreover, horses are also used in different sports like polo, tent pegging, etc. This is all because of the horse's phenotypic traits of athleticism. This trait in horses is due to the size and development of the skeletal muscular structure. Both genetic and environmental factors may influence racing traits in horses [2]. The horse's speed performance is genetically associated with the MSTN gene [3]. Myostatin protein, also known as GDF-8 (Growth differentiation factor 8) is encoded by MSTN gene. This protein belongs to the TGF-β (transforming growth factor beta) family of growth and differentiation factors [4, 5]. This protein restricts the development and growth of muscle fibers [6]. In recent studies, researchers found that the MSTN is linked to racing performance, growth of skeletal muscle, and carcass quality traits in different species including livestock animals, dogs, and horses [7]. Moreover, the Myostatin gene is a major determinant of racing performance because it also influences the proportion of fast twitch and slow twitch muscle fibers in

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horses [5, 8, 9]. The MSTN gene in *Equus caballus* is located on chromosome 18, accession ID NM_001081817.1 in assembly EquCab2.0 (GCF_000002305.2). It contains three exons and two intronic regions with a combined length of 4979 nucleotides. In horses, single nucleotide polymorphism (SNP) at region 18:66493737T>C (rs397152648) had been identified as a genetic marker to analyze the racing performance [10]. From the previous genetic studies, the wild-type genotype (T/T) is linked to horse's stamina and muscle development, heterozygous (T/C) genotype horses give better performance in middle distance races whereas homozygous mutant type (C/C) horses are top performers, fast-paced and short-distance sprinters [5, 11]. Furthermore, the studies also suggested that fast twitch glycolytic, type II-B muscle fibers provide short bursts of power in horses. Hence, the C/C horses have best racing performance and to be considered as sprinters. Whereas, in T/T horses the proportion slow twitch oxidative, type I muscle fibers are present which are required for showing better stamina performances [12]. The remaining horses with T/C genotype have both the muscle fibers in their body which shows mild racing performance [13].

The objective of current study was to investigate the MSTN gene variants associated with the racing performance among random-bred Pakistani horse population.

METHODS

Sample Collection and DNA Extraction

A heterogeneous horse breeds were chosen for investigating the association between the g.66493737T>C variant and its potential impact on racing performance and muscle development traits within the local equine population. Blood samples were meticulously obtained from 24 horses, with a distribution of 10 best performers and 14 controls. The sample of top performers was obtained from owners whose horses have consistently maintained top positions in past races, ensuring a representation of elite racing genetics within the study cohort. Collection was performed via jugular vein puncture using EDTA vacutainers, facilitated through consultations with horse attendants or owners. The collected samples were promptly stored at 4°C for subsequent analysis. Genomic DNA extraction from the horse blood samples was conducted using the GDSbio genomic DNA extraction kit, ensuring accuracy and reliability in the genetic analysis process.

Primer Designing

Utilizing the OligoCalc software (http://biotools.nubic.northwestern.edu/OligoCalc.html), we designed a set of five primers targeting the MSTN gene sequence (NM_001081817.1). Among these primers, three were selected for ARMS-PCR amplification: a forward common primer, along with reverse primers specific to the normal allele (N) and the mutant allele (M). These primers were designed from the 3'-end to facilitate the amplification of both wild-type and mutant variants of the targeted sequence. To enhance the specificity and efficiency of the ARMS-PCR assay, a secondary mismatch was deliberately introduced at the 4th nucleotide position from the 3' end of the reverse primers designed for both wild-type and mutant alleles. Additionally, we designed two supple mentary primers, one forward and one reverse, to amplify a region adjacent to our targeted sequence. This region served as an internal control (IC) to validate the accuracy and reliability of the amplification process (table 1).

Table 1: Primers Sequences and its Attributes

ARMS/ Internal Control (IC) Primers	Sequence (5'-3')	Tm (°C)	Length (bases)	Product Size (bp)
Forward Common	ATCTGTTATGTTT GGCTTTGGAATA	61.2	26	274
Reverse Normal	TATTAAGTAATCAGG TTATAATGCAC**T**AAA	60.5	30	
Reverse Mutant	TATTAAGTAATCAGG TTATAATGCAC**T**AA**G**	62.6	30	
Forward IC	CGGGTGCTCTC AACAATAGTA	57.9	21	685
Reverse IC	CAGATCTATTTTC AGGCTCTTTTAAC	58.3	26	

Tm = Melting Temperature; bp = base pairs

Subject Variant Amplification Through ARMS-PCR

The SimpliAmp thermal cycler (Applied Biosystems) was utilized to amplify both wild and mutant type variants. Each sample underwent two separate PCR reactions, with each reaction employing either the normal or mutant type ARMS reverse primer in combination with the common forward primer. Concurrently, an internal control region was amplified using a standard set of primers from an adjacent locus in each tube. A reaction mixture totaling 25µL was prepared, comprising 1µL of 50ng extracted genomic DNA, 1µL of 10.0 mM concentration of each primer (N or M reverse primers, forward common primer, forward & reverse IC), 1 unit of Taq polymerase, 2.5mM MgCl2, 2.5mM dNTPs, 1X Taq buffer, and PCR grade water. The PCR protocol consisted of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Subsequently, final extension step was conducted at 72°C for 10 minutes (figure 1).

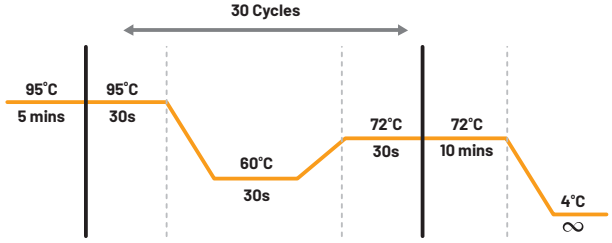

Figure 1: Thermal Cyclic Conditions of ARMS-PCR

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Statistical Association

Genotyping of g.66493737T>C locus was analyzed on our collected horse samples followed by Chi-Square association testing using PLINK data analysis toolset for genotypic and allelic frequencies along with their p-value calculation.

RESULTS

Variant Genotyping

A total of 24 horses were sampled, which includes (best performers n=10; controls n=14) Genotypic variability of g.66493737T>C variants was analyzed in Pakistani horse population. The ARMS-PCR results showed significant genotypic variability of g.66493737T>C locus which is associates with racing performance of horses as described in different studies [5]. Out of total 24 horses, 13 possess T/T homozygous wild genotype which may be considered to be the best for long-distance racing (stayer category), 10 horses showed T/C heterozygous genotype which may be considered as the best performers in middle-distance races (up to 8-12 furlongs) and only one horse had C/C homozygous mutant genotype which may be considered as the best performer in short distance races (sprinters category). As C/C genotype down regulates the expression of *MSTN* which increase the growth of muscle fibers. Consequently, more body-to mass ratio leads to better racing performance. Sampled horses are shown in figure 2B.

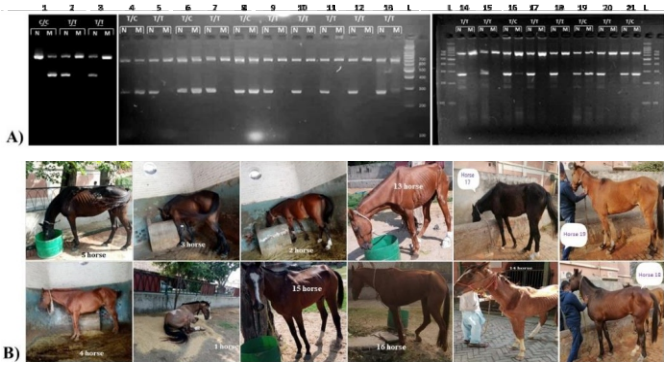

Figure 2: (A) ARMS-PCR amplification of targeted locus with laboratory case number from 1-21 and genotype, N and M represent amplification result of normal and mutant allele, L represent the Thermo Scientific GeneRuler 1 kb DNA Ladder, with internal control and amplified allele at 700bp and 300bp respectively (B) few sampled horses

Association Analysis

The statistical association along with both genotypic and allelic frequencies were calculated. The allele frequency distribution showed that the T/T homozygous wild genotype is common among Pakistani random-bred horses. Our screening results showed 54.16% of horse population were homozygous wild (T/T), while 41.66% were heterozygous (T/C) and the remaining were homozygous mutant with (C/C) genotype which was from the from top performer. Furthermore, ARMS-PCR results showed that "T" allele was more frequent than "C". The Chi-statistics was applied using PLINK data analysis toolset to calculate the p-value which was 7.832×10-6. The genotypic information, alternate allele frequencies in the best performers and controls horses are shown in table 2.

Table 2: Association Analysis of MSTN (rs397152648) in Pakistani Horse Population

Chr.	cDNA Variant NM_001081817.1	Protein Variant NP_001075286.1	Genotypic Information			Alternative Allele Frequency		p-Value
			Homozygous Wild TT/%	Heterozygous TC/%	Homozygous Mutant CC/%	Best Performer	Controls	
18	66493737T>C	Intronic	13/54.16	10/41.66	1/4.16	0.36	0.12	7.832×10^{-6}

DISCUSSION

MSTN gene has an impactful association with skeletal muscle mass development and fiber size in different species of horses as well as in other animals [13]. The genomic region of chromosome 18 containing MSTN gene was the highest ranked region in genome wide association study (GWAS) for the best racing performance [10]. In literature, many genetic variability studies in MSTN gene were performed on horses which showed that the mutation in MSTN gene locus (g.66493737T>C) is associated with racing phenotypes influencing its performance in different horse breeds [9]. In the current study, we investigated the effect of genetic variability on MSTN g.66493737T>C genotypes in Pakistani horse population. We performed analysis on 24 samples to find out the association with racing performance of the horses. No doubt, the sample size is small as compare to equivalent studies which were previously done by the researchers. Many scientists reported that this variant is associated with the racing performance and muscle development among different breeds of horses [14, 15]. Preceding studies showed a significant difference in genotypic and allelic frequencies associated with racing performance in thoroughbred and Anglo-Arabian breeds horse population [5, 16]. Previous researchers have reported the association between the MSTN polymorphism and racing performance in Anglo-Arabian horses. Results indicate that the SNP rs397152648, g.66493737T>C significantly influenced sport traits, suggesting its potential use in selection for improved sport performance in this breed [17]. Another study explored MSTN gene variants in Polish horse breeds and their association with height at the withers in Arabians. Five SNPs, including g.66493737T>C, were identified, with significant effects of g.66495696T>C on height observed in Arabian foals. These findings underscore the relevance of MSTN polymorphisms for morphological traits in Arabian

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horses [18]. In another research, the study examined the allelic frequencies of the g.66493737 SNP, along with other SNP marker positions, in MSTN gene of Nordic horse breeds. Significant haplotype effects were found on performance traits, such as tolt ability and BLUP values. The research involved a total of 25 horse breeds and four Donkey and Przewalski's horse individuals. These findings suggest potential functional implications of the studied MSTN gene variants on horse performance [19]. This study has demonstrated the statistical association analysis of genotypic and allelic frequencies on Pakistani horse population. In our random-bred horses, the results showed that the fourteen horses have homozygous T/T genotype, and the occurrence of T allele is relatively very high. On the other hand, prevalence of homozygous mutant (C/C) genotype was very rare in our horse population. The horses exhibiting the C/C and T/C genotypes were observed to be among the top performers in the study. However, it was noted that while both genotypes demonstrated high performance, the race efficiency of the T/C genotype horses was found to be comparatively lower than that of the C/C genotype. The C/C genotypes down regulates the expression of MSTN which increase the development of muscle fibers. Consequently, more muscle mass ratio leads to better racing performance in horses [20]. Our genotypic associations are linked to different research studies which are considered to determine the racing performance among the different horse breeds in European. Whereas the racing performance of our horse population was not recorded so for. In future, we are intended to record the racing performance with substantial sample size to identify more appropriate statistical association analysis.

CONCLUSIONS

The MSTN gene marker (g.66493737T>C) demonstrates variability within the Pakistani horse population, presenting a potential avenue for predicting racing performance and muscle development in indigenous horses. The prevalence of the homozygous wild-type (TT) genotype at 54% and the homozygous mutant-type (CC) at 4% indicates distinct genetic patterns. The observed variability in wild and mutant allele frequencies provides valuable insights for breeders, enabling marker-assisted crosses to achieve desired horse populations in the future. Furthermore, these findings have practical implications for various aspects of horse management, including selling, purchasing, training, selection/culling, and strategic breeding endeavors.

Authors Contribution

Conceptualization: RS
Methodology: RS, MHR, MOZ, WT, MD
Formal analysis: RS, MW
Writing-review and editing: MHR, MOZ, WT, MD, MW

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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