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Genetic Association of *PLAG1* Gene Variant 14:25015640G>T with Wither-Height in Pakistani Cattle

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ABSTRACT

The cow is one of the most valuable domesticated animals, producing milk, meat, fiber, hide and manure to serve humanity. Particularly, first two production traits are positively correlated with the physical characteristics of the animals e.g., wither-height, body size and skeletal frame. **Objectives:** The *PLAG1* is one of the many genes that has been significanlt associated with the aforementioned trait in many livestock and human species, so, genetic association of the 14:25015640G>T variant is being investigated in the current study. **Methods:** Genotyping of a total 50 cattles was conducted using ARMS-PCR technique followed by statistical hypothesis testing of the aforementioned variant using PLINK data analysis toolset. **Results:** Our findings depicted 24% of the sampled Pakistani cow population is homozygous wild-type for (GG), 12% homozygous-mutant (TT), while 64% found heterozygous (GT). Subject samples were obeying Hardy Weinberg Equilibrium (HWE) with x^2 (2,N = 50) = 10.39, p=0.049. Similarly, Chi-square association was also observed significant p = 1.267 x10^{-3} with minor-allele frequency of 0.60 and 0.28 in heighted (cases) and control cohorts respectively. Additionally, a positive odds-ratio of 3.85 is also evident that the subject variant is under-selection and showing the tendency of the mutant allele almost 4-times higher in cases vs control groups. **Conclusions:** This pilot scale study would be helpful to gain genetic insight of the subject variant in our sampled cow, however further functional studies with larger sample size is needed for validation and subsequent results can be disseminated to improve this valued trait of the indigenous cows for gaining maximum milk and meat production from this esteemed species.

INTRODUCTION

Cattle have been an integral part of human history. Initially, our ancestors sought this animal for sustenance and many other purposes including leather provision and draught purposes. Over the ~10,000 years, farmers have selectively bred and raised this animal particularly for enhanced meat and milk production [1]. As animal husbandry is one of the strong economic indicators in agriculture-based countries, particularly beef fattening, cattle is the most potential livestock species on a global scale. During the newnate calf judging process, the initial assessment parameter are birth weight (BW) [1] and calving ease which are the direct indicators of animal's growth and its carcass potential [2]. Cattle meat is also important for our health due to high content of iron, mineral and essential vitamins e.g., niacin, riboflavin and vitamin K, B6/B12 which reduces tiredness, fatigue, provide proteins and enhance the muscles strength [3]. The interaction of geographical regions, animal species and specific breeds along with different rearing systems give rise to distinct challenges in ensuring animal security. These challenges can have significant implications for both animal and human public health [2, 4]. Livestock is an important component of food and nutrition security owing to a number of reasons such as the high nutritional content of food products that are

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derived from animals. The recent history of cattle is characterized by variability in its body-size. Stature in the *Bos taurus* lineage was downsized by a factor of ~1.5 from the Neolithic to the Middle Ages and increased again only during the early modern ages [3, 5]. With heliotype analysis, it is clear that bovine *PLAG1* mutations have significant impacts on body size, weight and reproduction ~1000 years old derive allele that increased very quickly in its frequency in northwestern European *B. taurus* between the 16-18th centuries [6, 7, 8]. In livestock species, cattle stature is highly influenced by the Pleomorphic Adenoma Gene 1 *(PLAG1)*. The researcher first discovered this gene when they studied pleomorphic adenoma (PA) in human salivary glands that encodes zinc protein family, find in growth-related QNTs (9). The *PLAG1* gene regulates various cellular processes. Hence, it has been identified as one of the players in controlling body stature such as the development of skeletal-frame and overall body-size in cattle and other species [10, 11]. According to this, *PLAG1* gene-specific variants can affect the activity or expression influencing skeletal muscle growth by regulating the production of growth factors and hormones that are involved in bone formation and development of height in cattle [12, 13]. The *PLAG1* gene variant 14:25015640G>T has been chosen for the current investigation in order to genotype the cattle population in Pakistan and determine whether it is associated with wither-height. The indicated *PLAG1* gene variant is found in the *Bos_taurus*_UMD_3.1.1 (GCF_000003055.6) assembly with rs109815800 on Chr.14 ID AC_000171.1, transcript ID XM_005215432.2 (r.4098), and protein ID XP_005215489.1. Moreover, the stature of cattle contributes valuable insights for the advancement of more accurate genomic prediction models and to desiminate this variant in other cattle breeds and other livestock species as well upon the further functional validation studies.

METHODS

Sample Collection and DNA Extraction

This case-control study was conducted from March 2024 to March 2025. Ethical approval for this study was obtained from the Decodegenomics Research Training and Diagnostic Center (IRB Ref No. DG-adm-122). Animals for this investigation were owned privately by student families. Hair of 50 cattle was sampled to investigate the relationship of *PLAG1* gene with height and skeletal stature of Pakistani cattle. Two groups were created, one is a heighted-cohort (n = 25, wither-height >130cm at first parity) and the other as a control-cohort (n = 25, wither-height < 130cm at first parity) (Figure 1). Hair samples were stored in falcon tubes and kept at 4^{oC} for further usage. DNA were extracted from hair follicles using (Tiangenbioem Inc.) kit following the manufacturer suggested protocol. Although the analytical methodology uses a case-control comparison to assess genetic correlations, the cross-sectional sampling strategy was employed for data collection at a specific time point.

Figure 1: A few of Sampled Cows, Upper Row Animals are of Whether-Height >130cm at First Parity A), Lower-Row Animals are below this Threshold B).

Primer Designing for ARMS-PCR

OligoCalc and NetPrimer softwares were utilized to design ARMS primers from transcript ID XM_005215432.2. Five primers were designed, three of them are named reverse normal (N), reverse mutant (M) and forward common. These primers were designed for amplification of wild as well as mutant-type alleles. Reverse wild/mutant ARMS primers also contain an additional 3' end located secondary mismatch at 3^{rd} nucleotide position to enhance allele-specificity. As an internal control (IC), two more primers (forward and reverse) were also constructed for ensuring the PCR fidelity. The detail of all the primers is given in Table 1.

Table 1: ARMS-PCR Primers Sequences Details

ARMS/IC Primers	Sequence (5'-3')	Temperature (°C)	Length (bp)	Product Size (bp)
Forward Common	TCATGCAGAAAAAT -AACAAGCGAAA	59.2	25	300
Reverse Normal	CTAATGAGTTTTAT -GATTAGCCTAC	59.2	25	
Reverse Mutant	TACTAATGAGTTTT -ATGATTAGCCTAA	59.2	27	
Forward IC	TGAAAGCCAGA -GCCTGACTC	60.5	20	710
Reverse IC	GGTGAAATAATTGT-GCATAAGGAG	60.3	24	

DNA Amplification

An Applied Biosystems SimpliAmp thermal cycler was employed for the execution of the ARMS-PCR. Two separate PCR reactions were conducted using the normal (N) and mutant (M) type ARMS allele-specific reverse primers and a common forward primer alongwith two ordinary primers to amplify the genomic region as an internal control. The reaction mixture of the total volume of 12µL consists of 1µL of 50ng/µL genomic DNA, 10mM each primer, 0.05IU Taq polymerase, 2.5mM MgCl2, 2.5mM dNTPs, 1x buffer and molecular grade water. Five minute initial denaturation at 95°C was succeeded by 30 cycles of denaturation for 45 sec at 95°C, annealing for 30 sec at 60°C and extension for 45 sec at 72°C with the last extension at

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72°C for 10 mins and storage of 4°C for infinity.

Statistical Analysis

Hardy Weinberg Equilibrium (HWE) equation p2 +2pq +q2 =1 was applied to check whether the sampled population obeying the above equation or not, followed by Chi-square analysis $x^2 = \Sigma \frac{(0-E)2}{E}$ equation after determining the genotypic and allelic frequencies to evaluate the statistical association p-value. Moreover, the odd-ratio was also calculated using PLINK data analysis toolset. Article is available as bioRxiv preprint doi: https://doi.org/10.1101/2023.06.18.545456.

RESULTS

In the present work, *PLAG1* variant 14:25015640G>T (rs109815800) was genotyped and identified to be variable in Pakistani cattle population. We genotyped 50 samples (n=25 with wither height >130cm, and n=25 with wither height <130cm) on first parity. After analyzing this data statistically, we identified 12 animals are homozygous-wild type (G/G), 6 homozygous-mutant (T/T) and 32 heterozygous (G/T) in the total sampled population. Whereas in control group 11 cattle were homozygous-wild and 14 were heterozygous whereas none of the animal was found to be homozygous mutant. Again in heighted-cohort 01, 18 and 06 cattle are homozygous wild-type, heterozygous and homozygous-mutant respectively as indicated in Figure 2.

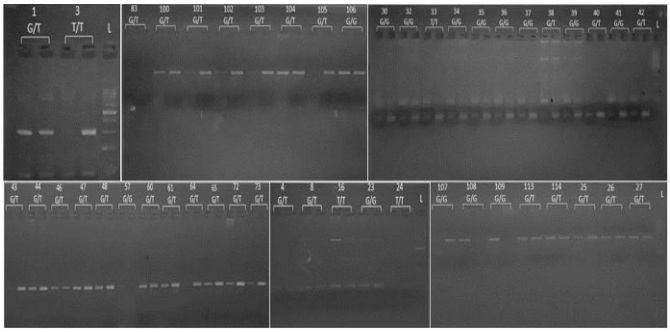

Figure 2: ARMS-PCR amplification of the Targeted Variant within Sampled Cows, Internal Control and Allele-Specific Product Size of 700 and 300bp

The Chi-square test for Hardy-Weinberg Equilibrium (HWE) in the population sample provided a p-value of 0.049 which is lower than the commonly utilized cutoff ($p < 0.05$), thereby suggesting deviation from HWE. In addition, genetic association was also inferred which yielded mutant allele frequencies of 0.60 in the cases and 0.28 in the controls with p – value 1.267 x10^{-3} which indicated significant association with wither-height phenotype. Likewise, odds-ratio (OR) of 3.85 was also found which states prevalence of odds/mutants is roughly ~4-times greater in cases than controls as reported in Table 2.

Table 2: Statistical Association of *PLAG1* Gene Variant 14:25015640G>T with Pakistani Cows Wither-Height Phenotype

No. of Samples	Chromosome	cDNA Variant XM_0052 15432.2	Protein Variant XP_005 215489.1	Genotypic Information			Alternative Allele Frequency		p-Value and (OR)
				Wild Type (GG)/%	Hetero (GT)/%	Mutant Type (TT)/%	Cases	Control	
50	AC_000171.11 4:25015640	rs109815800 (G>T)	p.=	12/22	32/64	6/12	0.60	0.28	1.267×10^{-3} (3.85)

The multiple sequence alignment of *PLAG1* gene was performed using Clustal Omega a web-based MSA tool to find out the conservancy status of the genotyped variant in total 13 mammalian species of pig, cattle, deer, donkey, horse, monkey, human, chimpanzee, tiger, wolf, beer, dog and fox. The high level of conservancy of the subject variant was found in all species except the tiger (Figure 3).

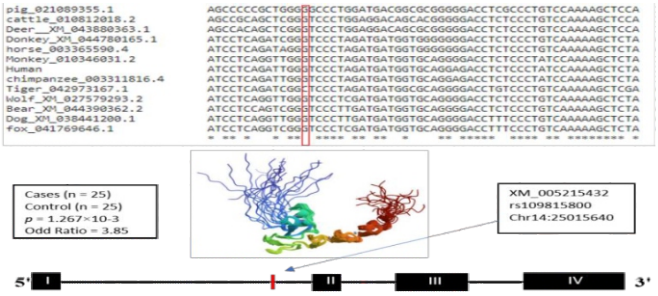

Figure 3: MSA of Genotyped Variant within Different Mammalian Species

DISCUSSION

Based on the animal's wither height at initial parity, the cattle in this study are split into two groups. Due to added weight and blood flow in the udder, an animal's height at a certain age is a good indicator for estimating its future growth potential and milk yield. Additionally, skeletal-frame can forecast the fattening patterns in cattle breeding stock. However, estimated breeding values (EBVs) from weight records give the best prediction of weight and performance in offspring from breeding stock [7, 14]. Animal height, as a measure of maturity, has also been found to be an informative predictor of feedlot performance. The bigger animals mature faster and gain less body fat compared to smaller animals. A helpful measure for communicating and comparing height is the frame score based on hip height at a given age. Frame score is a widely accepted system for communicating the magnitude of an animal's skeleton. Most animals will have the same frame score at all ages, even though their actual

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height increases with age. Body type scores, valid for nearly all cattle breeds with minimal regional differences, were created at the University of Wisconsin in the USA and vary from 1 to 11 [15, 16]. The pilot study presents evidence that the *PLAG1* gene is under selective pressure for height recovery in Pakistani cattle. There is a particular mutation that is involved in the trait since the geographic distribution of the mutation differs from that of height [17]. Genotyping revealed 12 homozygous wild-type, 6 homozygous mutant, and 32 heterozygous cows in case and control groups, in support of the under-selection hypothesis. Multiple sequence alignment conservative analysis in 13 mammalian species supported the evolutionary relevance of the locus [18]. *PLAG1* has been identified previously as an important regulator of body size in cattle and other animals [19]. In contrast to humans, animals have less complex genetic designs for height. *PLAG1* has also been implicated in height and body weight in cattle and horses and selective sweeps in pigs and dogs [16, 20]. These observations underscore the gene's function in mammalian development and the possible potential of natural variants for breeding. Our findings contribute to this evidence base, demonstrating a significant relationship between the *PLAG1* variant and wither height in Pakistani cattle, in accordance with observations in other livestock.

CONCLUSIONS

Wither-height with the *PLAG1* gene variant (rs109815800) in Pakistani cattle is significantly associated with a p-value of 1.267×10^{-3} and odds ration of 3.85. Further functional studies coupled with validation in larger cohorts could support the implementation of marker-assisted breeding strategies that enhance cattle stature and improve their production in terms of milk and meat.

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Authors Contribution

Conceptualization: RS
Methodology: RS, MN, SZ, SF
Formal analysis: RS, MN, SZ, SF
Writing and editing: MN, SZ, SF

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

Conflicts of Interest

The authors declared no conflict of interest.

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