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Identification and *in silico* impact assessment of missense mutations in key genes associated with hypoxic stress in Changthangi goats

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In order to enhance breeding programs and boost genetic features related to disease resistance, productivity and adaptability, it is essential to predict harmful mutations in livestock animals. The genetic adaptations enabling Changthangi goats to thrive in high-altitude environments are essential for their survival in conditions marked by hypoxia, extreme temperatures and oxidative stress. Therefore, in this study, missense mutations were mined within key candidate genes, namely *CXCL9*, *MRPL22* and *SDS* involved in hypoxic stress, in Changthangi goats to assess their potential effects on the structural integrity of the proteins. Two SNPs (rs653275315, rs654804090) were identified with significant impacts on protein structure and stability, while one SNP (rs644425445) was categorized as benign. The SNP rs654804090 introduced changes in hydrophobicity, impairing protein stability, whereas rs653275315 affected conserved domains critical for function. Structural analyses revealed that the identified mutations disrupted protein folding, stability and molecular interactions, particularly in highly conserved residues, underscoring their evolutionary significance. Phylogenetic conservation analysis further highlighted the adaptive relevance of these mutations, particularly in regions subjected to environmental stressors. Our findings reveal potentially detrimental mutations in the genes *CXCL9, MRPL22* and *SDS*, which may contribute to the development of genetic screening tools for selective breeding initiatives especially in high-altitude settings where genetic resistance to environmental stress is essential.

Keywords: *CXCL9*, Ladakh, *MRPL22, SDS,* SNPs, Transcriptome

Goat breeds adapted to high-altitude environments exhibit remarkable physiological and genetic adaptations that enable them to thrive in conditions of low oxygen availability, extreme temperatures and rugged terrain. These breeds possess unique traits that include enhanced oxygen transport mechanisms, efficient energy metabolism and robust thermoregulation[1]. At high altitudes, hypoxia-inducible factors (HIFs) play a crucial role in regulating the expression of genes involved in oxygen sensing, erythropoiesis and angiogenesis, allowing these animals to maintain oxygen homeostasis[2-4]. Morphologically, they often have larger lung capacity and higher red blood cell counts to optimize oxygen delivery to tissues. Additionally, their metabolic pathways are fine-tuned to ensure energy efficiency and resilience against oxidative stress[5]. Understanding the genetic basis of these adaptations not only sheds light on evolutionary biology but also provides insights for improving livestock resilience in changing climatic conditions. Changthangi goats are well-adapted to the extreme conditions of the cold desert region of Ladakh, thriving at elevations above 4000 meters where temperatures fluctuate between +30°C and -30°C[2]. Their resilience is partly attributed to genetic adaptations that enhance their ability to cope with oxidative stress, a common challenge in high-altitude environments. These adaptations are likely mediated by specific genes that regulate cellular responses to hypoxia and oxidative damage, enabling these goats to maintain physiological stability under harsh environmental conditions[6,7]. Transcriptomic studies have identified several hub genes that are potentially linked to high-altitude adaptation in Changthangi sheep and goats[2-4,8]. Many of these genes are associated with mechanisms that mitigate oxidative stress, which is a critical challenge in high-altitude environments. These genes likely play key roles in maintaining cellular homeostasis by regulating oxidative damage and enhancing resilience to hypoxic conditions[5]. Detecting polymorphisms in

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these genes can provide valuable insights into the genetic basis of high-altitude adaptation, enabling the identification of genetic markers associated with resilience to hypoxia and oxidative damage.

Single-nucleotide polymorphism (SNP) or a single-point mutation in the genome, is the substitution of one nucleotide for another in the genome sequence[9]. While some SNPs are found in coding areas, other SNPs may occur in noncoding regions. Silent mutations are SNPs that occur in noncoding regions of genes and have no impact on the protein sequence or the gene product[10]. On the other hand, when the mutation results in a change in one or more amino acids in the relevant protein sequence it is known as a missense mutation[11]. Missense mutations can affect the proteins involved in the trait of interest. By identifying these mutations, breeders can select goats with beneficial genetic traits. Identifying missense mutations helps in understanding the genetic basis of desirable traits. This knowledge can be used to develop breeding programs aimed at enhancing specific traits, leading to better and more consistent production performance in goats. Therefore, this study aimed to mine missense mutations in the *longissimus thoracis* muscles of Changthangi goats within candidate genes *associated with hypoxic stress*, namely *CXCL9*, *MRPL22* and *SDS* to assess their potential effects on the structural integrity of the proteins.

Materials and Methods

Extraction of variants

Quality assessment of the reads in the samples was done with FASTQC tool[12]. The raw reads were analyzed with CLC Genomics Workbench[13]. The reads having Phred score ≤30 and adapter content were removed using Trim Reads tool of CLC genomic Workbench. The filtered reads were mapped against the *Capra hircus* reference assembly ARS2 using Map Reads to Reference tool of CLC. These Mapped reads were then treated with Variant Detection tool of CLC workbench to detect the SNPs. The detected variants were further processed with Link Variants to 3D Protein Structures tool of CLC Workbench to detect the functional consequences of these variants. The methodological approach followed in this study is depicted in (Fig. 1).

Prediction of missense variants and their effect on molecular dynamics

To anticipate the detrimental impact of missense mutations on protein function PolyPhen 2.0 and the SIFT programme of Variant Effect Predictor tool was used. This programme helps in sorting intolerant from tolerant by using sequence homology to forecast the effect of a mutation on the protein function. Mutations with a score less than 0.05 were labeled as deleterious[14].

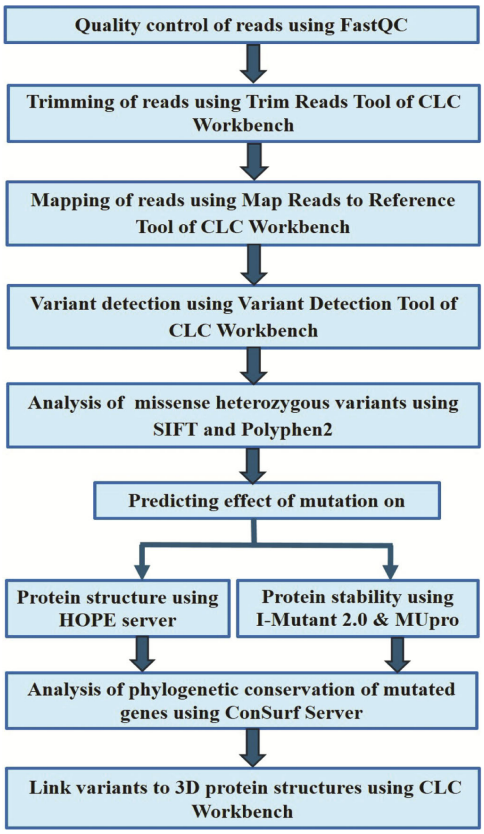

Fig. 1 — Flow chart of the methodological approach

Functional annotation

The selected SNPs were annotated using the Variant Effect Predictor (VEP) Tool[15]. The SIFT value generated by the VEP was checked against each of the SNP.

Effect of mutations on the structure and stability of protein

The single point mutations were analyzed using the next-generation web server Project HOPE[16] (Have (y) Our Protein Explained) accessible at https://www3.cmbi.umcn.nl/hope/, PolyPhen 2.0 (Polymorphism Phenotyping v2) was utilized to assess the effect of the identified mutation on the structure and function of the protein[17]. I-Mutant 2.0 and MUpro were used to predict the effect of mutation on the stability changes in the protein[18,19].

Analysis of Phylogenetic Conservation of Mutated Genes

ConSurf, a web tool for finding functional areas in proteins by examining the evolutionary dynamics

of Amino Acid mutations among homologous sequences, was used to analyze the conservation prediction of the altered protein sequences[20]. This tool categorizes the protein's amino acid residues on a scale of 1 to 9, where 1-3 indicates varied, 4-6 indicates average, and 7-9 indicates conserved or highly conserved regions. The variations deemed most harmful were those that fell within the conserved region[20].

Results

The transcriptome data of *longissimus thoracis* muscles of Changthangi goats (SAMN12500993 to SAMN12500996 under the Project PRJNA62481) was used for the analysis. On an average 130,045 variants were detected across all samples, these variants included SNPs, indels, MNVs and replacements. The average SNV discovered in the dataset was 111,674. One lakh six thousand eight hundred and fifty number of SNPs were selected that were heterozygous in all the samples examined. From this dataset, missense SNPs were subsequently filtered, focusing on those with the potential to cause amino acid changes that could impact protein structure or function. Heterozygous SNPs that were common to all samples were selected for further analysis. Three SNPs were selected that exhibited missense mutations; details of these SNPs are given in (Table 1).

Effect on stability of the Protein

Predicting the structural effect of mutations on protein stability is crucial in understanding how alterations impact structure and functions of the protein. A mutation can either stabilize, destabilize or have no significant effect. MUpro results predicted that all the mutations decreased the stability of their corresponding proteins (Table 2). I-Mutant 2.0 showed that mutation rs654804090 increased the stability of their corresponding proteins whereas mutations rs653275315 and rs644425445 decreased the stability of their corresponding proteins with the reliability index values ranging between 0 and 9 (Table 2).

Phylogenetic Conservation

The mutation rs653275315 had a conservation score of 7 (Fig. 2A). In contrast, the mutations rs654804090 and rs644425445 occurred at variable residues with conservation scores of 3 (Fig. 2B & C).

Prediction of Structural effects of mutations:

The predicted phenotypic effects of the mutation rs654804090 (N203T) suggest that the wild-type residue is the preferred secondary structural form. The local conformation is predicted to exhibit slight instability due to the preference of the mutant residues for a different secondary structure. The wild-type residue demonstrates greater hydrophobicity compared to the mutant residue, which is also smaller in size (Fig. 3A). This reduction in size may lead to a loss of stabilizing interactions. Furthermore, the mutation introduces a residue with increased hydrophobicity at this position. This alteration could result in the disruption of hydrogen bonds, potentially hindering proper protein folding and stability.

The mutation rs644425445 (N280K) occurs within a domain classified as "Tryptophan synthase beta chain-like PALP" (UniProt), introducing an amino acid with distinct physicochemical properties that may disrupt the domain's functionality. Structurally, the altered residue is located on the surface of a domain with an undefined function and does not interact with any recognized domains within the

Table 1 —Summary of the prediction results of missense mutations using SIFT and PolyPhen 2.0

Gene	SNP ID	Nucleotide change	Amino Acid Variant	SIFT		PolyPhen 2.0	
				Score	Prediction	Score	Prediction
CXCL9	rs653275315	T →C	Met66Val	0.04	deleterious	0.146	benign
MRPL22	rs654804090	T →G	Asn203Thr	0.05	deleterious	0.522	Possibly damaging
SDS	rs644425445	G →T	Asn280Lys	0.03	deleterious	0.001	benign

Table 2 — Comparison of MUpro and I-Mutant 2.0 for prediction of effect of variants on stability of the protein

SNP ID	MUpro						I-Mutant 2.0		
			SVM Method		Neural Network Method				
	Delta G	Prediction	Effect	Confidence score	Effect	Confidence score	DDG Prediction(kcal/mol)	RI	Stability
rs653275315	-1.52	Decrease	Decrease	-0.18	Increase	0.59	-1.84	9	Decrease
rs654804090	-1.69	Decrease	Decrease	-0.65	Decrease	-0.89	0.36	4	Increase
rs644425445	-1.38	Decrease	Increase	0.07	Increase	0.56	-1.05	3	Decrease

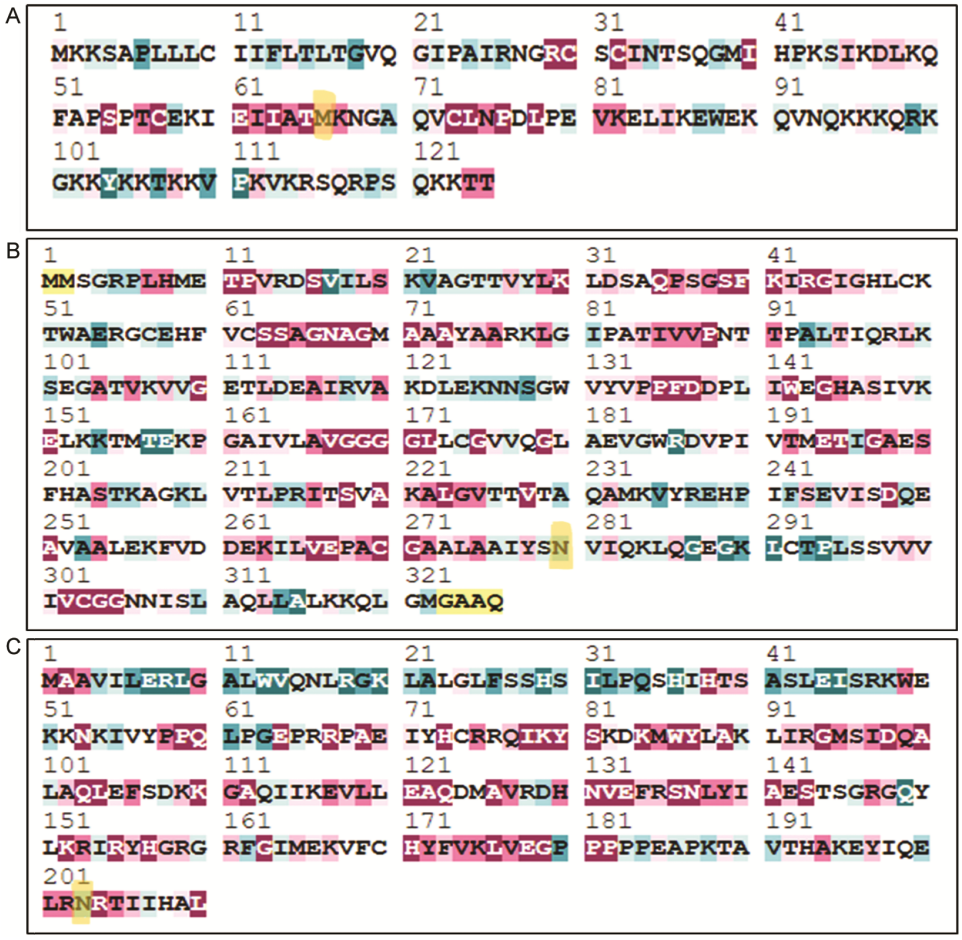

Fig. 2 — (A) Evolutionary conservation analysis of amino acid residues of *CXCL9*; (B) Evolutionary conservation analysis of amino acid residues of *SDS*; and (C) Evolutionary conservation analysis of amino acid residues of *MRPL22*

analyzed structure; however, it may still affect interactions with other molecules or domains. The mutant residue differs significantly from the wild-type in both size and charge, with the larger mutant residue potentially causing steric hindrance and altering molecular interactions (Fig. 3B). Additionally, the charge difference may lead to electrostatic repulsion between the mutant residue and adjacent residues. As the residue is surface-exposed, the mutation could disrupt interactions with other molecules or regions of the protein.

The mutation rs653275315 (Met66Val) occurs in Chemokine interleukin-8-like domain annotated in Uniprot. The mutation introduces an amino acid with different properties, which can disturb this domain and abolish its function. The mutant residue is smaller than the wild-type residue and is located near a highly conserved position. The smaller size of mutant residue might lead to loss of interactions (Fig. 3C). The original wild-type residue and newly introduced

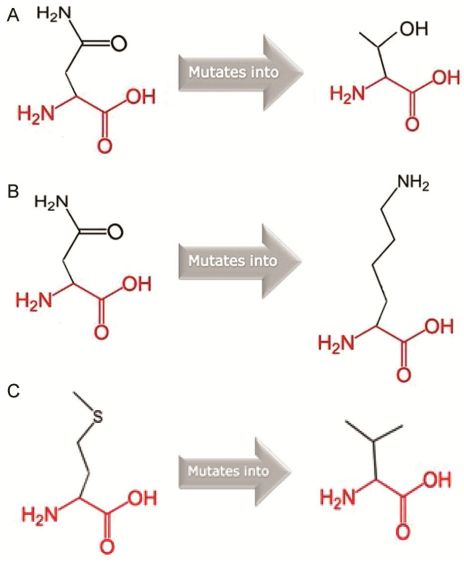

Fig. 3 — (A) Schematic structure of original and mutant amino acid of rs654804090; (B) Schematic structure of original and mutant amino acid of rs644425445; and (C) Schematic structure of original and mutant amino acid of rs653275315

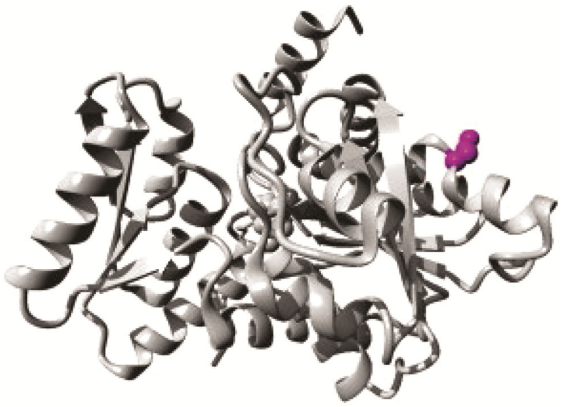

Fig. 4 — Structural alteration of Protein by the mutant residue K280

mutant residue differ in properties like size, charge and hydrophobicity-value. Figure 4 provides a structural overview, depicting the protein in grey and the mutated residue's side chain in magenta, represented as small spheres.

Discussion

The study of missense mutations in candidate genes in the skeletal muscle transcriptome of Changthangi goats provide significant insights into the genetic adaptations that enable these animals to thrive in high-altitude environments. The focus on missense SNPs in genes such as *CXCL9, MRPL22* and *SDS* associated with hypoxia, will help in elucidating the potential impact of these variations on the physiological resilience of Changthangi goats, particularly in extreme high-altitude conditions. *CXCL9* is a chemokine gene involved in inflammation and immune responses, with its expression regulated by hypoxia-inducible factors (HIF-1α) under low oxygen conditions[21,5]. It plays a role in immune pathways like Toll-like receptor signaling, cytokine interactions, and pathogen recognition. Under hypoxic stress, *CXCL9* influences immune responses and inflammation, which are critical for adaptation to high-altitude environments[22,23]. Hypoxia affects signaling pathways, including cytokine responses, and may influence immune evasion in certain pathogens. Hypoxia-inducible factors (HIFs) play a crucial role in regulating the expression of genes like *MRPL22*, which are vital for the cellular response to low oxygen levels. These adaptations are critical for survival in hypoxic environments, such as high altitudes[24]. Transcriptome profiling has revealed significant changes in gene expression under hypoxia, indicating that genes like *MRPL22* are involved in metabolic adjustments that help cells withstand low oxygen conditions[25]. The *SDS* gene, encoding serine dehydratase, plays a key role in metabolic adaptation to hypoxia, contributing to glycine, serine, threonine, and cysteine-methionine metabolism. Under hypoxic conditions, metabolite levels, such as glutathione and cystathionine, fluctuate, affecting cell survival and oxidative stress responses[26]. These metabolites are crucial for maintaining redox balance, with reductions in glutathione and cystathionine observed in low-oxygen environments[27].

The results of this study reveal several missense mutations in the Changthangi goat *longissimus thoracis* muscle transcriptome that are predicted to have significant effects on protein function. Among the three SNPs selected for detailed analysis, two (rs653275315 andrs654804090) were predicted to be deleterious by both SIFT and PolyPhen 2.0, while rs644425445 was identified as benign in some predictions. The prediction of deleterious effects suggests that these mutations could potentially impair the structural and functional integrity of the corresponding proteins, thereby affecting muscle function, energy metabolism and stress resistance[28,29]. Similar detrimental mutations have also been detected in the bovine *CMAH*, human *HLA-G1* isoform and the *RASSF5* gene[30-32].

All four mutations decreased the stability of their respective proteins, with varying degrees of reliability. Mutations rs653275315 and rs654804090 were predicted to destabilize the proteins significantly. These findings suggest that while some mutations may have negative impacts on protein function, others might contribute to structural adaptations that allow the protein to maintain stability under stressful environmental conditions. Such structural changes could be important for the ability of goats to adapt to high-altitude environments where oxygen supply and temperature regulation are critical[33]. The structural analyses further revealed that some mutations could result in significant changes in the protein's stability and interaction potential. For example, the mutation rs654804090 (N203T) was predicted to cause slight instability due to changes in secondary structure and hydrophobicity. Mutations in secondary structures and hydrophobic residues can destabilize proteins by disrupting hydrogen bonding networks, altering packing density, and affecting structural rigidity, leading to improper folding, reduced stability[34,35]. Similarly, rs644425445 (N280K) introduced a larger and charged residue that

could disrupt the protein's molecular interactions, potentially affecting its function in muscle tissue. This aligns with Freundtand Linke[36] study which demonstrate that structural alterations in muscle proteins like titin can impair their mechanical properties, particularly elasticity and tension generation that are crucial for proper muscle function.

Phylogenetic conservation analysis indicated that the mutation rs653275315 occurred at highly conserved residues, suggesting its evolutionary importance[37]. These highly conserved residues are critical for the proper functioning of the protein, and mutations in such positions are more likely to have detrimental effects on protein function[38]. While the rsIDs themselves are species-specific and do not have direct orthologs, the associated gene *CXCL9* has been shown to respond to hypoxic stress across different species. Studies indicate that chronic hypoxia increases *CXCL9* expression in primary rat brain astrocytes[39] and hepatocarcinoma cells[40.] These findings suggest that *CXCL9* plays a conserved role in hypoxia-related immune responses. On the other hand, mutations rs654804090 and rs644425445 occurred at more variable residues, suggesting that they may not be as essential for the basic function of the protein but could still influence its interaction with other molecules or contribute to adaptive phenotypic traits. Yang and Bielawski[41] demonstrated that SNPs in non-conserved regions can significantly affect protein stability and function, emphasizing their role in molecular adaptation.

Limitation of the study

While this study provides valuable insights into the genetic basis of high-altitude adaptation in Changthangi goats, further research is needed to confirm the functional consequences of these mutations. Experimental validation, such as protein expression studies and functional assays, will be crucial to understanding the precise role of these mutations in muscle biology and high-altitude resilience. Additionally, a larger sample size with more diverse goat populations could provide a more comprehensive understanding of the genetic diversity and adaptive potential in this breed.

Conclusion

The identification and analysis of missense SNPs in the transcriptome of Changthangi goats offer important insights into the molecular underpinnings of their high-altitude adaptation. The knowledge gained from this study can help in pinpointing genetic markers that are associated with traits such as muscle development, energy metabolism and thermoregulation. Understanding the impact of these mutations on protein structure and function will enable breeders to develop targeted strategies to improve livestock performance, particularly in regions where environmental stress poses a challenge. These findings highlight the importance of integrating genomic data into selective breeding practices, particularly in high-altitude environments where genetic resilience to environmental stressors is crucial. The ability to select for beneficial mutations, such as those that enhance oxidative stress tolerance or improve protein function in harsh conditions, can contribute to the development of more robust livestock populations.

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Conflict of interests

All authors declare no conflict of interests.

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