

## IDENTIFICATION OF VARIATIONS IN THE CODING REGION OF MYOSTATIN (MSTN) GENE OF THALLI AND PAK-KARAKUL SHEEP BREED IN PAKISTAN

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### ABSTRACT

Marker assisted selection (MAS) is a potential tool to genetically enhance the traits of economic importance in livestock production. One of the gateways to MAS is the identification of SNPs for the desired genes. The aim of the present study was to detect SNPs in myostatin (MSTN) gene which has a negative role in growth and skeletal muscle development. Therefore, polymorphisms of the exon no 1, 2 and 3 of MSTN gene were studied in Thalli (n=10) and Pak-karakul (n=10) sheep breeds. The genomic DNA was isolated and amplified by using PCR. The amplicons were purified and sequenced. After PCR all samples were sent to Singapore for 1<sup>st</sup> base laboratory for further purification and sequencing. Sequencing analysis was done using Codon Code Aligner and MEGA 6 software's. Mutations were observed at only exon no 1 in 50% (5/10) Thalli sheep (heterozygous G>T =3/5, synonymous =2/5) and 20% (2/10) Pak-karakul sheep (only synonymous T mutations) at 3995bp. The two alleles (A and B) were found having all the three combinations of genotypes i.e. AA, AB and BB. Allele frequencies (A+B) were 0.2 and 0.8 in Pak-karakul and in case of Thalli it was 0.5 in each. The data from the present study suggests that SNPs in MSTN gene are present. Furthermore, scope of the present study may be enhanced to establish a marker for improved mutton production through conducting association studies at large scale.

**Keywords:** Myostatin (MSTN); Thalli and Pak-karakul sheep; Polymorphism; Pakistan; Mutton

### INTRODUCTION

Myostatin (MSTN) belongs to the TGF- $\beta$  superfamily of signal transduction proteins, which demonstrates a significant role in muscular growth and lipid metabolism (An *et al.*, 2011). It plays a major role as a adverse regulator in the development and growth of skeletal muscles. The function of MSTN gene was explored by producing the mice without MSTN gene, as mice without MSTN gene had higher muscular growth than mice having MSTN gene (Whittemore *et al.*, 2003). Different forms of MSTN gene exist in many livestock species and each form of gene has been associated with some productive trait (Gan *et al.*, 2008). The polymorphism in this gene has been determined in various breeds of sheep (Zhou *et al.*, 2008) and goat (Han *et al.*, 2015). Ovine MSTN gene is 4991bp in size and is present on chromosome no. 2 (Boman *et al.*, 2009). Single Nucleotide Polymorphisms (SNPs) within the coding region of myostatin gene have been found to be associated with double muscling (Hadjipavlou *et al.*, 2008). Moreover the myostatin sequence analysis of double muscled European breed revealed 7 DNA sequence polymorphisms and concluded that five of them were responsible in modulating the functions of protein (Joulia-Ekaza & Cabello, 2007). Genetic characterization of Boer goat for myostatin gene also confirmed SNPs associated with productive and reproductive traits (Zhang *et al.*, 2012). Strategies to improve profitability in sheep industry mainly focus on the reproductive traits (Wang and Wu, 2006; Yu L *et al.*, 2007). However, mutations occur naturally in sheep which affect muscular growth and development. The best documented mutation for muscle development in sheep is callipyge (CLPG), which causes a postnatal muscle hypertrophy that is localized to the pelvic limbs and loin (Noelle, 2005). Similarly variations in MSTN gene has been studied by various scientists (Tay, 2004) and interestingly these are being used as a selection marker for improved muscular yield commercially (Zhang *et al.*, 2012).

However, to our knowledge, there is no study available in the literature investigating the variations in myostatin gene of Thalli and Pak-Karakul sheep in Pakistan. Therefore, the aims of this study were to: find out the variations in myostatin gene of Thalli and Pak-Karakul sheep and design the effective breeding plans of Thalli and Pak-Karakul sheep in Pakistan for enhanced mutton production through increased growth rate.

### MATERIALS AND METHODS

The data from adult male sheep of Thalli (n=10) and Pak-karakul (n=10) at Small Ruminants Research and Development Centre, RakhKhairwala, District Layyah, Pakistan were collected. A total of five ml blood sample was collected from jugular vein from each animal in 15ml falcon tube containing 200 $\mu$ l EDTA. Genomic DNA was extracted from whole blood samples by using modified method (Sambrook *et al.*, 1989). The extracted genomic DNA was stored at -20°C. Agarose Gel-electrophoresis technique was used to confirm the extracted DNA. New Primers were designed using sequence of myostatin gene available at NCBI public gene bank database (Gene ID: 443449). Primers were designed (Table 1) for amplification of Exon 1, 2 and 3 of ovine MSTN gene by online free software (Primerfox), available at website (<http://www.primerfox.com/>).

Gradient PCR for each exon was done to find the actual annealing temperature of each primer. Initial denaturation was done at 95°C for 3 min and then cycle started at 95°C for 30sec. Annealing comprised of 35cycles, each cycle of 45 seconds at 50-60°C and extension was done at 72°C for 45sec. Final extension was carried out at 72°C for 10 min and then amplicons were stored at 4°C. After finding the annealing temperature (52°C and 59°C) at gradient PCR, PCR was done. First initial denaturation was done at 95°C for 3min and then cycle started at 95°C for 30sec. Annealing comprised of 35cycles, each cycle of 45 sec at 58°C of exon 1, 3 and 50°C for exon 2. Extension was done at 72°C for 45sec and final extension was carried out at 72°C for 10 min. Then amplicons were stored at 4°C.

A small quantity (3µl) of PCR product was added into wells of 1% agarose gel for electrophoresis. To indicate the base pair length, 1 Kb ladder in one well of the gel was added. A charge of 80 volts was passed through the gel for approximately 40-60min. This causes the negatively charged DNA to travel towards the positively charged electrode. After PCR all samples were sent to

Singapore for 1<sup>st</sup> base laboratory for further purification and sequencing. Sequencing analysis was done using Codon Code Aligner and MEGA 6 software's.

**Table 1** Design of Oligonucleotide primers of MSTN gene of Thalli and Pak-Karakul sheep species.

Exons	Oligonucleotides primers	Amplicons (bp)	Annealing temp.	Melting temp.
Exon 1 F.	CAAGTTGTCTCTCAGACTGG			
Exon 1 R.	ACACTAGAACAGCAGTCAGC	545bp	58°C	60°C
Exon 2 F.	ATAGATTGACATGGAGGC			
Exon 2 R.	ATAAGCACAGGAAACTGG	551bp	50°C	52°C
Exon 3 F.	TAATGACTCCTTGCGGTAGG			
Exon 3 R.	TACTCTAGGCTTATAGCCCC	548bp	58°C	60°C

**RESULTS**

In an elementary phase of this research, Gradient and standard PCR were done by using the DNA extracted from the blood samples. The PCR product was run on agarose gel for visualizing the predicted size. The amplification of total DNA of blood samples yielded the PCR fragments of 545 bp, 551 bp, and 548 bp as it was expected size for Exon no 1, 2 & 3 respectively. In exon no 1 G/T mutation was observed in three Thalli sheep and two Pak-karakul sheep samples (fig 1).

**Polymorphism in MSTN gene**

Ten samples of each breed were sequenced to check the polymorphisms in Thalli and Pak-karakul sheep population. Heterozygous loci were observed at the position number 3995th of accession number DQ530260.1.T/G heterozygous position was observed in three Thalli sheep and at the same position G/T mutation in two Pak-Karakul sheep breed at exon 1 region while at exon 2 and 3, no any mutation were observed. Two types of alleles were observed, mutant allele was labeled as “A” and wild type as “B” (Table 2).

**Calculation of Gene and Genotypic Frequency**

Gene and genotypic frequency was calculated with the help of popgene software. Gene frequency of Exon no. 1 was different because mutation was observed in Thalli sheep breed. While, no mutation detected in case of Exon 2 and Exon 3. So according to Hardy Weinberg law, Gene frequency was same in case of no mutation.

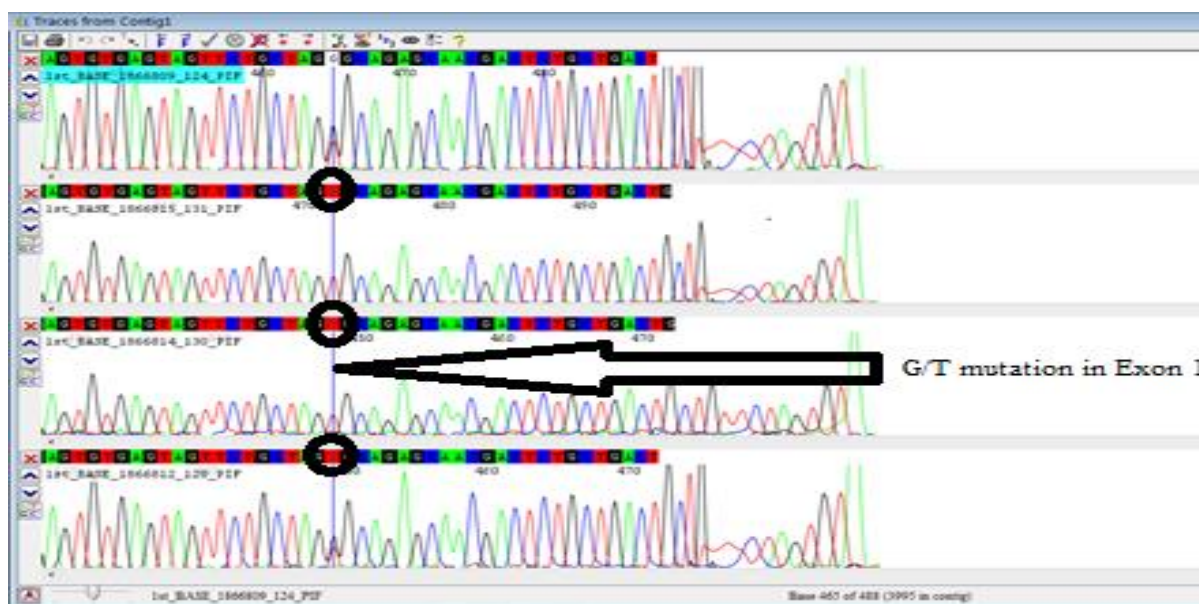
**Calculation of MSTN gene frequency**

Exon 1 was polymorphic in Pak-karakul sheep breed; it had mutation at 3995 region of MSTN gene. The following calculations were used for MSTN gene frequency measurement.

Total no of animals of each breed 10

The formula of gene frequency is:  $(A+B)^2 = A^2 + B^2 + 2AB$

Calculated Gene frequency and expected genotypic frequency is shown in the table 3 which is calculated by Hardy Weinberg law. These results showed that heterozygosity exists in local sheep breeds of Pakistan.



**Figure 1** Sequencing results and comparison of samples of Exon 1 read with CodonCode Aligner.

**Table 2** Mutations in exons in Thalli and Pak-karakul sheep breed

SR.NO	Breed	Exon 1	Mutation	Exon 2	Exon 3
1	Thalli	G/T	Heterozygous	++*	++
2	Thalli	G/T	Heterozygous/Synonymous	++	++
3	Thalli	G/T	Heterozygous/Synonymous	++	++
4	Pak-karakul	T	Homozygous/Synonymous	++	++
5	Pak-karakul	T	Homozygous/Synonymous	++	++

Note: where ++ shows the wild type or no mutation.

**Table 3** Gene and genotypic frequency.

	Gene Frequency		Genotypic Frequency		
	A*	B*	AA	AB	BB
Pak-karakul	0.2	0.8	0.04	0.32	0.64
Thalli	0.3	0.7	0.09	0.42	0.49

\* Polymorphic allele, +wild type allele

## DISCUSSION

The MSTN gene encodes a protein which acts as a negative regulator for the muscular mass in mammalian species. Its role has been described for growth of muscle. Despite the undesirable effects of double muscling, the characterization of the polymorphisms that results in double muscling has created an opportunity to exploit the phenotype in livestock breeding using optimal breeding systems (Keele and Fahrenkrug, 2001). The variations in the MSTN gene have been extensively studied and reviewed in various small and large ruminants in the world (Zhou H et al., 2008), but it has never been studied in sheep breeds of Pakistan.

To better understand the distribution of genetic variation in ovine MSTN, this investigation explored diversity in 2 commercial sheep breeds from Pakistan. An extended region of the gene spanning nucleotide no. 3995 in the promoter region was analyzed. The present study was based on sequence analysis of the non-coding region of the sheep myostatin gene, four SNPs were identified in the 5' promoter region, 5'-UTR and 3'-UTR and seven new reported SNPs were identified in the exons. All the SNPs were found at the position 3995 in exon no.1. The main emphasis of present research shifted to analyze the SNPs in the regulatory region (putative promoter and UTRs) because they are most likely to influence the meat traits by modulating the level of MSTN expression. Piedmontese and Belgian Blue cattle breeds have phenotypically the doubled muscular appearance with decreased bone mass, connective tissue and fat. An obviously distinct muscular hypertrophy, usually known as double muscling, occurs with high frequency in these breeds (Esmailzadeh et al., 2008). Hence, SNPs of the MSTN gene could influence the functional structure of MSTN protein and has direct impact on muscle growth (Hadjipavlou et al., 2008). It was found that two SNPs in the MSTN gene have considerable link with muscle depth of commercial Charollais sheep (Yu et al., 2007) and two SNPs in the 5' regulatory region of the MSTN gene were associated with untimely growth character in Yorkshire pigs (Esmailzadeh et al., 2008). SNP (F94L) in the MSTN gene affecting growth, reproduction and beef quality traits of *Bostaurus* (Kambadur et al., 1997; McPherron and Lee, 1998). The heterozygosity was observed only in Thalli sheep breed. The Thalli breed has maximum body weight but in the case of karakul breed that has high body weight the only T mutation was found. G/T transition was observed only in exon 1 of myostatin region. So it can be concluded that heterozygous position G/T and T mutations can significantly facilitate muscle growth.

## CONCLUSION

It is concluded from the present study that polymorphism is present in both local Pakistani breeds (Thalli and Pak-Karakul). The outcome of the current study may be applicable in identifying markers and designing breeding plans of both sheep breeds for enhanced mutton production through analyzing association studies at large scale.

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