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Molecular Evaluation of the Myostatin Gene in Four Iranian Native Goat Breeds Did not Demonstrate any Association with Twining Rate

Research Article

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ABSTRACT

The objective of this study was to evaluate myostatin (MSTN) gene by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and sequencing methods in four goat breeds including Mohabadi, Markhoz, Lori and Bital breeds. A573 bp fragment of MSTN gene was amplified by PCR. A new 5 bp deletion in 5`UTR (206TTTTA) of the MSTN gene was directly identified by sequencing. The AA and AB genotypes were observed in all above breeds, while the BB genotype was found only in Mohabadi breed. The AA genotype had the highest genotypic frequency in all breeds. On the other hand, the BB genotype in Mohabadi goat breed had the lowest genotypic frequency. The A allele frequency was higher than that of the B allele in the four breeds investigated in this study. The A allele frequency significantly differed between two (litter size) subgroups in Mohabadi breed. This difference in the allele frequency was not significant in Lori and Mohabadi breeds. Therefore, odds ratios statistics (ORs) showed no significant differences between genotypes for twining rate. Evaluation of MSTN gene 5`UTR region demonstrated the presence of two TATA boxes (important putative regulatory elements) which are located in the promoter region (position 32-36, 56-60) and one E-box (position 42-48) motifs. In conclusion, our results showed that the 5`UTR region polymorphism cannot be a key factor in the occurrence of twining in the above native goat breeds. It seems that more studies in large populations are needed to investigate the effect of MSTN gene on twining with more focus on other mutations in MSTN gene or other genes.

KEY WORDS genetic diversity, goat, myostatin, twining.

INTRODUCTION

Twining trait is one of the important factors affecting profitability of goat production enterprises. However, improvement of reproduction traits by traditional selection methods has been proved to be slow because they have low heritability and expressed only by sexually mature females leading to long generation intervals (Zhang *et al.* 2011). Molecular techniques like the candidate gene approach provide a good breeding tool that can accelerate improvement of goat twining early in life (Zhang *et al.* 2009). Furthermore, for goat as a polytocous species with limited twining, the marker-assisted selection (MAS) can potentially play a much more important role than that in other species due to the fact that the growth traits are greatly associated with twining. Myostatin (MSTN) gene or growth differentiation factor-8 (GDF-8) is associated with increased skeletal muscle mass in the several mammalian species such as mice,

cattle, sheep, horse and goat. Myostatin is a member of the transformation growth factor (TGF)-β, encompasses super family, a large group of secreted growth and differentiation factors that play important roles in regulating development, tissue homeostasis and reproduction (Rong *et al.* 2007). Myostatin is synthesized as a precursor and upon proteolytic processing gives an N-terminal latency associated peptide, termed myostatin propertied or LAP-fragment and a smaller mature peptide at the C-terminus (Miar *et al.* 2014).

Myostatin gene in the goat has three exons and two introns and located on the chromosome 2. It has been shown that the coding region has approximately 6.2 kbp length and separated by a small intron (1.8 kbp) and a larger intron (2.4 kbp) (Geoffrey, 2009).

It has been recently described that MSTN gene is expressed as a negative regulator of skeletal muscle mass in mice, as well as, as a regulator of responding to androgen and glucocorticoids for reproduction cycle (Ma *et al.* 2001).

Myostatin receptor is primarily responsible for reproduction development and can be a potential candidate gene for fertility (Sandi and Rose, 1994). Moreover, MSTN plays an important role in the regulation of reproductive system in livestock (Hu *et al.* 2009).

A single nucleotide polymorphism (SNP) in the 3`untranslated region (3`UTR) (previously named g+6223G>A) was shown to create or destroy putative miRNA target sites and hence affects muscularity (Han *et al.* 2010). Few SNPs in the promoter region of the swine MSTN gene were associated with muscularity, growth and meat quality traits (Stinckens *et al.* 2008). Also, three SNPs in the 5`regulatory region (5`UTR) and two SNPs in the 3`UTR region of chicken MSTN gene have been identified (Xianghai *et al.* 2007). In addition, polymorphisms of 5`UTR and 3`UTR regions from MSTN gene have been detected in Iranian (Kamangar *et al.* 2014) and Lori sheep (Sepahvand *et al.* 2013), respectively. A few studies have been investigated MSTN gene in goats. The study of MSTN polymorphism using PCR-RFLP on four Chinese goat breeds showed a deletion in 5`UTR region of this gene (Zhang *et al.* 2012) and reported high genetic diversity in those populations. In another study, several SNPs and a deletion (TTTTA) in MSTN gene were identified in seven Indian goat breeds (Singh *et al.* 2014). In addition, a significant association of TTTTA deletion in 5`UTR of MSTN gene with twining in goat has also been reported (Khani *et al.* 2014).

Mohabadi, Markhoz, Lori and Bital breeds are reared in different locations in Iran for different purposes such as meat, milk and cashmere production. This study was performed to evaluate the MSTN gene for the first time in these goat breeds, and also to investigate its potential association with the twining rate.

MATERIALS AND METHODS

DNA samples and data collection

Based on litter size (LS) in kidding, a total of 210 blood samples were collected from four Iranian native breeds including 60 Markhoz goats (30 with LS=1 and 30 with LS>1), 60 Mohabadi goats (30 with LS=1 and 30 with LS>1), 60 Lori goats (30 with LS=1 and 30 with LS>1) and 30 Bital goats (LS>1). The genomic DNA was extracted using Diatom DNA Prep 100 extraction kit according to instructions of the manufacturer.

Primer design and PCR condition

A pair of primers was designed using Oligo 5 software to amplify a 573 bp fragment of MSTN gene 5`UTR region (Gen Bank accession no. EF591039) as the following: forward 5`-GGAGCAAGAGCCAATCACAGA-3` and reverse 5`-AATGACCGTTTCCGTCGTAAC-3`.

The PCR amplification reactions were carried out in a final volume of 25 µL contained 50-100 ng genomic DNA, 0.25 µ*M* of each primer, 1 units of Taq DNA polymerase, 2.5 m*M* $MgCl_2$, 2.5 µL 10X reaction buffer (10 m*M* Tris-HCl, pH 8. at 25 ˚C, 50 m*M* KCl) and 2 m*M* dNTPs. The cycling protocol was initial denaturation at 94 ˚C for 5 min, followed by 35 cycles of denaturing at 94 ˚C for 30 s, annealing at 63 ˚C for 30 s and extension at 72 ˚C for 45 s, followed by a final extension at 72 ˚C for 10 min. PCR products were separated by electrophoresis on 1% agarose gel and stained with ethidium bromide.

Enzyme digestion of PCR products

The 573 bp amplified fragment of MSTN gene 5`UTR region was digested using *DraI* restriction endonuclease (recognition sequence: 5`.TTTAAA/.3`), which was selected using DNA star software and Webcutter (http://rna.lundberg.gu.se/cutter2/). The PCR products were digested at 37 ˚C for 12-14 h with digestion reaction volume of 15 µL included 7 µL of the PCR products, 1.4 µL enzyme buffer, 6.6 µL ddH_2O and 6 unit of restriction enzyme. The digested products were visualized by electrophoresis on 3% agarose gel and stained with ethidium bromide.

Sequence analysis using software tools

Four samples of each genotype were randomly selected for sequencing. The primers used for sequencing were the same as those for the PCR reaction. Moreover, the sequences obtained were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/SNP/index.html).

Statistical analysis

Pop Gene software (version 32) was used to obtain allelic and genotypic frequencies, population index and to test Hardy-Weinberg (H-W) equilibrium in four Iranian goat breeds. The logistic regression model (SAS, 2002) was used for evaluating association of MSTN genotypes with twining trait as follows:

$Y_{ij} = \mu + B_i + M_j + e_{ij}$

Where:
Y_{ij}: dependent variable.
μ: population mean.
B_i: fixed effect of the i^{th} breed.
M_j: fixed effect of the j^{th} genotype.
e_{ij}: random residual effect with an expected value of 0 and normal distribution.

RESULTS AND DISCUSSION

A 573 bp fragment of Caprine MSTN gene was successfully amplified by PCR as shown in Figure 1. A new 5 bp deletion in 5`UTR (206TTTTA) of the MSTN gene was directly identified by sequencing.

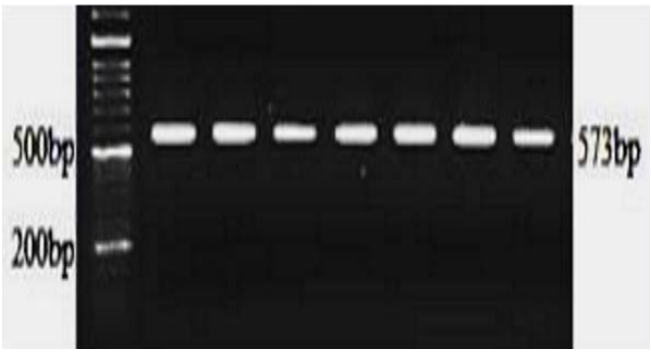

Figure 1 PCR products of myostatin gene 5`UTR region in four Iranian goat breeds

The wide-type allele (with TTTTA) named B was digested by *DraI* endonuclease and produced two 210 bp and 363 bp fragments (Figure 2). The *DraI* endonuclease did not digest mutant allele (named A) and resulted in 568 bp fragment (Figure 2). Possible combination of these alleles resulted in AA, AB and BB genotypes. Genotypic and allelic frequencies, as well as H-W equilibrium test in these four goat breeds are shown in Table 1. The AA and AB genotypes were observed in four studied breeds while the BB genotype was observed only in Mohabadi goats. The AA genotype had the highest genotypic frequency in four breeds in contrast to Mohabadi goat breed in which the BB genotype had the lowest genotypic frequency.

Thus, as shown in Table 1, the frequency of the A allele was higher than the B allele in four breed investigated in this study. The allelic frequency of the A allele was 0.85, 0.94, 0.91 and 0.88 for Mohabadi, Markhoz, Lori and Bital breeds, respectively.

The MSTN locus was not in H-W equilibrium in Mohabadi goat population ($P<0.05$) while H-W equilibrium was observed in Markhoz, Lori and Bital goat populations ($P>0.05$).

The genotypic frequency showed a tendency AA > AB within breeds except for Mohabadi breed, which had the frequency of genotype AA > AB > BB. Effective numbers of alleles (Ne) were 1.3213, 1.1234, 1.1803 and 1.2596 in Mohabadi, Markhoz, Lori and Bital breeds, respectively.

Sequence analysis of MSTN gene 5`UTR region

The 573 bp amplified amplicon which encompasses 5` upstream sequence and partial CDS (coding exon I) was sequenced (Figure 3). The obtained gene sequence was aligned with Nubian, Mauto, Sannen, Angora, *Ovos areis*, *Bos taurus* and *Sus scrofa* using CLC Genomics (version 4.0), whereas there was no variation among the sequences of Iranian goat and other goat breeds. A different sequence was obseved for goat and other species investigated in this study. Sequencing analysis of the MSTN gene promoter showed that this fragment contains several kinds of seed-specific promoter motifs (Figure 4).

Association of the polymorphism with twining

Allelic frequencies of MSTN gene in two subgroups per breed have been compared in Table 2. The frequency of the A or B alleles was significantly different ($P<0.05$) between LS= 1 and LS > 2 subgroups only in Mohabadi breed. This diference was not significant in Markhoz or Lori population ($P>0.05$).

The results of ORs for twining rate among different genotypes are presented in Table 3. There was no significant ORS between genotypes for all breeds ($P>0.05$). The amounts of ORS and Chi-Square statistics for the twining rate were 1.14 and 0.17 for the AA/AB ratio, 1.39 and 0.58, for the AA/BB ratio and 0.71 and 0.58, for the AB/BB ratio in Mohabadi breed. The ORS of the twining rate in 5`UTR region of MSTN gene were estimated 1.16 ($P>0.05$) for the AA genotype in comparison with the AB in Markhoz breed, 1.25 ($P>0.05$) for the AA genotype in comparison with the AB in Lori breed and 1.19 ($P>0.05$) for the AA genotype in comparison with the AB in Bital breed.

Molecular evaluation of MSTN gene 5`UTR region

In this study, a 573 bp fragment including 5`UTR and exon 1 regions of goat MSTN gene was amplified and sequenced.

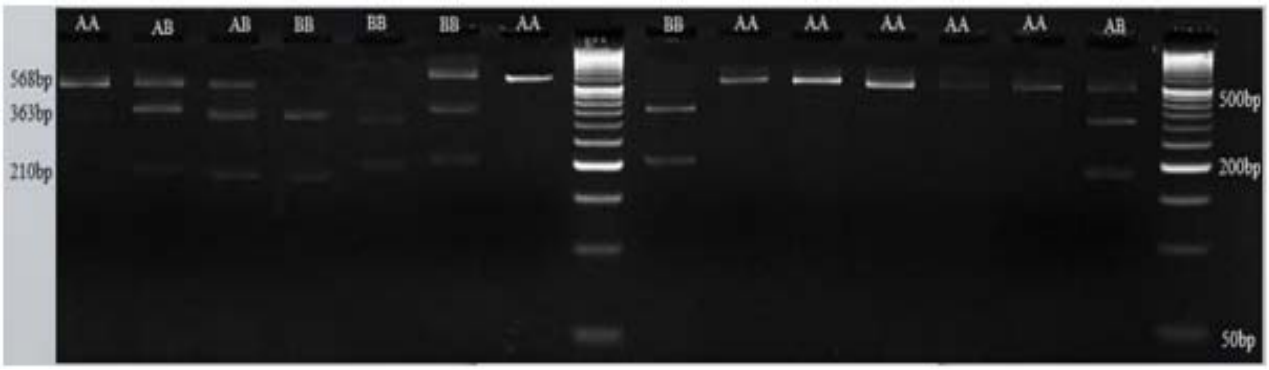

Figure 2 Three genotypes observed using PCR-RFLP method in four Iranian goat breeds

Table 1 Genetic diversity of studied region of myostatin gene in Iranian goat populations

Genetic diversity	Mohabadi	Markhoz	Lori	Bital
A allelic frequency	0.85	0.94	0.91	0.88
B allelic frequency	0.15	0.06	0.09	0.12
χ2-test	4.03 (P<0.05)	0.19 (P>0.05)	0.44 (P>0.05)	0.44 (P>0.05)
Observed heterozygosity	0.1833	0.1167	0.1667	0.2333
Expected heterozygosity	0.2452	0.1108	0.1541	0.2096
Nei index	0.2432	0.1099	0.1528	0.2061
Ne	1.3213	1.1234	1.1803	1.2596
Shannon index (I)	0.4080	0.2224	0.2868	0.3602

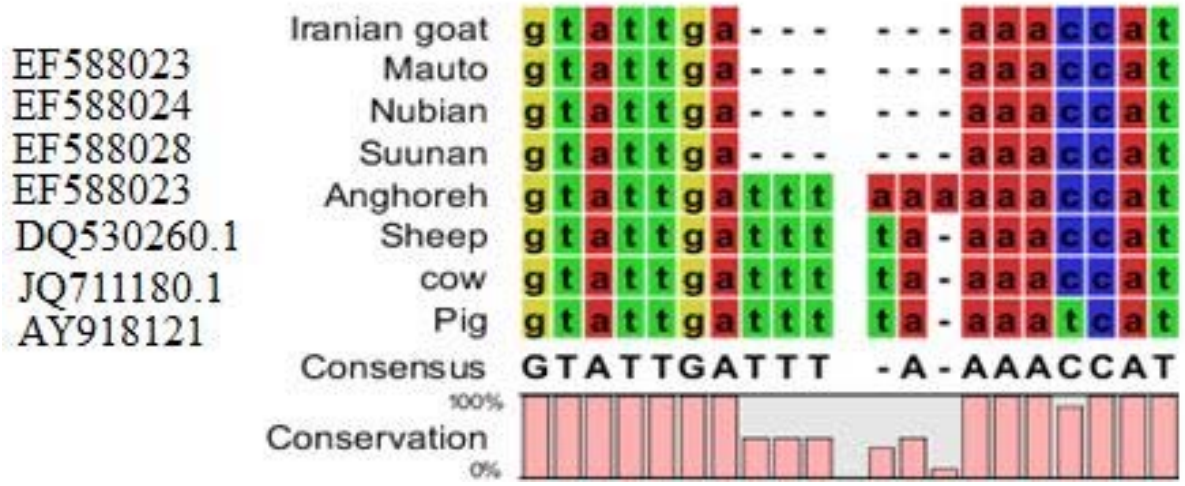

Figure 3 Alighnment of myostatin gene 5`UTR sequences in Iranian goat breed along with African Nubian goat (EF588024), Mauto (EF588023), European Sannen goat (EF588028), Asiatic Angora goat (EF588023), *Ovos areis* (DQ530260.1), *Bos taurus* (JQ711180.1) and *Sus scrofa* (AY918121)

These sequences exhibited more than 98% identity among four Iranian goat breeds. It has been recently reported that sequences of 5`UTR region of goat MSTN gene reveal a high degree of conservation (>90%) with those of cattle and sheep (Singh *et al.* 2014). The region contains two TATA-box motifs, which are located in the promoter region of the MSTN gene (Position 32-36, 56-60) as shown in Figure 4.

Our results of mutational analysis in the individual E-box motifs (position 42-48) showed that E1 had important effects on the activity of the goat MSTN promoter.

In another study, the mutational analysis of the individual TATA-Box and E-Box motifs showed that E1, E2, E3, E4 and E5 had effects on the activity of MSTN gene promoter in sheep, cattle and goat (Du *et al.* 2013).

Since, the TATA-box and E-Box motifs are key regulator factors for gene expression and transcriptional initiation (Dall'Olio *et al.* 2010) and any change or mutation in these areas may be a reason to change gene expression in myogenesis and probably reproductive process (Du *et al.* 2007).

For example, a bioinformatics study on Italian horse breeds showed that one SNP (g. 156T>C SNP) located in

the conserved TATA-box motif is associated with variability of morphology traits (Dall'Olio *et al.* 2010).

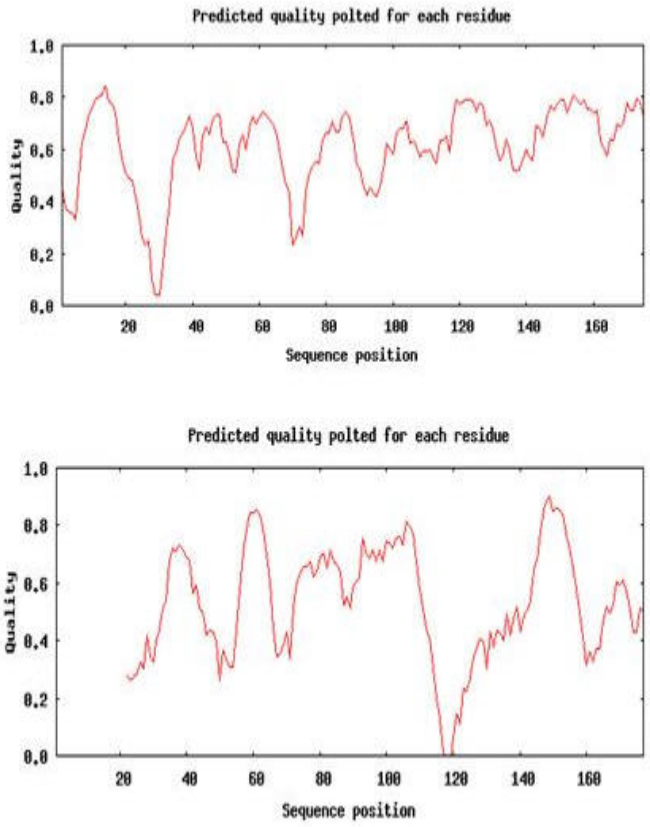

Figure 4 Sequence analysis of 529 fargment of the myostatin promoter region in Iranian goat breeds
1: TATA Box (position 32-36 and 56-60)
2: E Box1 CACTTG (Position 42-48)
3: Deletion TTTTA / (position 180-185)
4: MSTN gene start codon (position 190)

Therefore, some motifs in goat MSTN gene may be important for transcriptional regulation. More experimental methods are needed to be adopted to further testify roles and mechanisms of these motifs in myogenesis and reproductive processing.

Genetic diversity of different populations

In the present study, a 5 bp TTTTA deletion located in 5`UTR region of MSTN gene was observed in four Iranian goat breeds. This is in agreement with previous researches which reported the presence of same deletion in Boer, Chinese and Indian goat breeds (Zhang *et al.* 2012).

In this study, frequency of A mutant allele was higher than that of B allele and this pattern was relatively the same (0.85-0.94) among Iranian goat breeds, which is in agreement with other researches such as seven native Indian goat breeds (Singh *et al.* 2014) and four Chinese goat breeds (Zhang *et al.* 2012).

The MSTN polymorphism in Sanjabi sheep breed using PCR-RFLP has been investigated by Soufy *et al.* (2009). They observed three MM, Mm and mm genotypes and also estimated the highest frequency for m allele in intron 1 of the MSTN gene (Soufy *et al.* 2009).

Mohabadi breed showed the highest average heterozygosity (0.2432), Shannon's information index (0.4080), observed heterozygosity (0.1833) and expected heterozygosity (0.2452) among all populations studied. This result was in agreement with the researches on five Iranian goat breeds (Mohammad Abadi *et al.* 2009) by ISSR markers and also in Taleshi, Tali, Markhoz and Najdi goats (Sadeghi *et al.* 2009) by microsatellite, as well as, by RFLP method in Boer and Chinese goat (Zhang *et al.* 2012). In our study, the deletion locus was in H-W disequilibrium ($P<0.05$) in Mohabadi population, but Markhoz, Lori and Bital populations showed H-W equilibrium ($P>0.05$). The H-W disequilibrium may be related to mutation, mating and migration in the population.

Most of the variation in the genome occurs in the form of SNPs. Under favorable condition, the highest genetic diversity is an indicator of the history of the different breeds. Therefore, it is very useful for investigating population relationship, correlation of genetics distance and phylogeographical structure of Iranian goats. Additionally, this diversity in populations makes breeders able to use other breeds in crossbreeding programs in order to decrease inbreeding in native goats.

Effect of MSTN gene on twining rate

Some studies on Iranian sheep breeds has been conducted in order to find genes affecting twining rate. No polymorphism has been found in $FecX^G$ and FecB genes in Lori-Bakhtiari sheep breed (Amiri *et al.* 2007) as well as Baluchi sheep breed (Moradband *et al.* 2011). However, Moradband *et al.* (2011), for the first time, reported a significant association of GDF-9 polymorphism with twining rate in Balouchi sheep breed.

A few studies on genes related to twining in Iranian goats have been also performed such as FecB gene in Najdi goat breed (Mohammadi and Alimahmoudi, 2011) and BMP15 gene in Jabal Barez red goat breed (Alinaghizadeh *et al.* 2010). These researchers did not observed polymorphism in the above genes. Furthermore, Hua *et al.* (2008) observed none of polymorphism of ovine fecundity major genes (FecB and FecX) in goats with high superovulation. They demonstrated that major genes affecting twining rate in sheep and goat may be different.

Our results for comparing allelic frequency of MSTN gene between LS= 1 and LS > 2 subgroups (Table 3) showed a significant difference of the A allele only in Mohabadi goat breed ($P<0.05$).

Table 2 Allelic frequency of the myostatin gene in two subgroups per breed

Breed	Mohabadi		Markhoz		Lori		Bital	
Allelic frequency	A	B	A	B	A	B	A	B
LS[1]= 1	0.83[a]	0.17[b]	0.93[b]	0.07[b]	0.90[b]	0.30[b]	088	0.12
LS > 1	0.88 [b]	0.12[a]	0.95[b]	0.05[b]	0.93[b]	0.07[b]	-	-

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
LS: litter size in kidding.

Table 3 Estimation of odds ratios and Chi-Square statistics for twining rate among observed genotypes

Locus	Breed	Odds ratios (ORs)	Chi-Square	Pr > ChiSq
	Mohabadi		0.52	0.7719
	AA / AB	1.1405	0.17	0.6768
5`UTR	AA / BB	1.3939	0.58	0.4465
	AB / BB	0.7174	0.58	0.4465
	Markhoz		0.16	0.6871
5`UTR	AA / AB	1.1648	0.18	0.6682
	Lori		0.48	0.4872
5`UTR	AA / AB	1.2500	0.56	0.4528
	Bital		0.19	0.6656
5`UTR	AA / AB	1.1948	0.20	0.6508

So far, allele effect attributable to MSTN gene has not been reported previously in Iranian sheep and goat. The only study on association of this gene with twining in goat showed that the mutant allele in 5`UTR region has a significant association (ORS=0.57, P<0.1) with twining trait in 150 Iranian Markhoz goats (Khani *et al.* 2014). However, ORS statistics estimated in our study revealed that there were no significant differences for twining between different genotypes in four breeds (P>0.05). One of the reasons for this difference in results can be limited number of samples in our study.

Although we did not observe significant effect of MSTN gene on twining rate in the native goats, MSTN is the essential hormone for mature development which is responsible for the twining (Ríos *et al.* 2002). In addition, Ríos *et al.* (2002) reported that MSTN hormone is a key factor for Caprine production and reproduction, as well as developmental processing in human and mammals (Ma *et al.* 2001).

MSTN hormone, after secretion into the bloodstream and muscle cells, increases P21 (inhibitor of cycling cell cycle) via binding to the receptor serine/threonine activin kinase (Stinckens *et al.* 2008). Therefore, MSTN gene is the main regulator of postnatal growth, which results in stimulating anabolic processes such as cell division, skeletal growth, protein synthesis and fertility rate (Joulia-Ekaza and Cabello, 2007). So, it seems that mutation in MSTN gene can be effective on metabolism, production or reproduction in goat.

However, few researches have investigated association of MSTN polymorphisms with reproduction traits in goats and further studies are needed to verify this association in large populations.

CONCLUSION

According to our results, mutations identified and some putative regulatory motifs in MSTN sequences using molecular techniques are effective solutions to survey double muscle phenotype, twining rate and other production and reproduction traits, which help breeders to make precise decisions for managing and selecting the best animals. Our study did not show a significant effect of MSTN on twining so that more studies are needed to investigate presence of other MSTN gene mutations in these native goats in order to better understand effects of this gene on twining rate or growth traits.

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