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Full Length Research Paper

Allelic polymorphism of 'Makoei' sheep myostatin gene identified by polymerase chain reaction and single strand conformation polymorphism

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Myostatin, a transforming growth factor-beta (TGF-β) super family member, has been well documented as a negative regulator of muscle growth and development. Myostatin with 376 amino acids is synthesized as a precursor protein. In this study, polymorphism of myostatin gene in Iranian 'Makoei' sheep breeds was investigated by polymerase chain reaction and single strand conformation polymorphism technique (PCR–SSCP). Genomic DNA was isolated from the blood of 92 sheep. A 417 bp myostatin intron 1 segment was amplified by standard PCR, using the locus specific primers. Four different SSCP patterns, representing four different genotypes, were identified. The frequencies of the observed genotypes were 0.413**, 0.293, 0.130, and 0.163 for AD, AC, AE, and BC, respectively. Allele frequencies were 0.4185, 0.0815, 0.2283, 0.2065 and 0.0652 for A, B, C, D and E. Observed heterozygosity (Hobs) value was 0.7192. The chi-square test showed significant (P<0.05) deviation from Hardy-Weinberg equilibrium for this locus in studied population.**

Key words: Myostatin gene, polymerase chain reaction (PCR), single strand conformation polymorphism technique (SSCP), *Ovis aries*.

INTRODUCTION

Myostatin, a transforming growth factor-beta super family member, acts as a negative regulatory factor in skeletal muscle mass development, by inhibiting the Myo5 and MyoD factors, which are related to the mechanisms of differentiation of precursor cells into myoblasts (McPherron and Lee, 1997). Myostatin protein is first synthesized in skeletal muscle as a 52 kDa propeptide and then prototypically processed at the conserved RSRR site to produce a 40 kDa latency- associated peptide and a 13 kDa mature peptide which then forms a homodimer (26 kDa) to bind to its receptor(s) for its biological function. Ovine myostatin gene is located on chromosome 2 and a single nucleotide polymorphism in the 3 un-translated region of the gene has been shown to affect muscularity in sheep (Clop et al., 2006; Kijas et al., 2007).

The myostatin gene consists of three exons and two introns in all species studied (Bellinge et al., 2005). The effects of the myostatin gene were first described in mice, where loss of myostatin expression in knock-out mice was associated with both an increase in the number of muscle fibers (hyperplasia) and fiber size (hypertrophy).

The muscles of myostatin knock-out mice weighed about twice as much as those of wild-type mice. Later, an extreme form of muscularity (double muscling) seen in the Belgian blue and Piedmontese cattle breeds was shown to result from mutations in the coding region of the

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myostatin gene (Kambadur et al., 1997; McPherron and Lee, 1997; Wiener et al., 2009).

The product of myostatin gene is biologically inactive and has 273 amino acids. In sheep, quantitative trait loci (QTL) studies showed that myostatin gene had a major effect on muscular development in Belgian texel (Marcq et al., 2002) and on muscling depth in New Zealand Romney sheep (Hickford et al., 2009), Norwegian white sheep (Boman et al., 2009), Charollais sheep (Hadjipavlou et al., 2009) and New Zealand texel sheep (Johnson et al., 2009).

In total, 23 ovine myostatin gene single nucleotide polymorphisms (SNPs) have been reported (Clop et al., 2006; Kijas et al., 2007; Zhou et al. 2008). However, while SNPs provide some indication of the structural diversity of a gene, extended haplotypes are typically more informative, especially if they encompass all or most of the coding region. To date, three allelic variants have been reported in a 473-bp section of the exon I - intron I region (Zhou et al., 2008) and four haplotypic variants have been constructed through the coding sequence using SNPs positioned at 41, +4036 and +6223 (Kijas et al. 2007).

Polymorphism in intron I of ovine myostatin gene identified using single-strand conformational polymerphism analysis and revealed that five allelic variant in a 417 bp in New Zealand Romney sheep (Hickford et al., 2009). However, when Kijas et al. (2007) included two microsatellites that flanked myostatin gene, 20 haplotypes were described. The objective of this study was to characterize potential variation at the ovine myostatin gene in 'Makoei' sheep breeds using polymerase chain reaction and single-strand conformational polymorphism (PCR–SSCP) analysis.

MATERIALS AND METHODS

Sheep, blood sample collection and genomic DNA extraction

'Makoei' sheep breeds examined in this study were fat-tailed sheep with medium body size, white colour with black spots on face and feet. They are kept in east and west Azerbaijan provinces of Iran and their main products are meat and wool (Saadat-Noori and Siah-Mansoor, 1992).

Blood samples (approximately 2 to 3 ml) were obtained from 92 unrelated 'Makoei' sheep breeds originating from different parts of west Azerbaijan and stored in ethylenediamine tetraacetic acid (EDTA)-coated tubes. Genomic DNA was extracted from 0.3 ml blood using the genomic DNA purification kit (Fermentas, EU) according to manufacturer's instructions.

Quality and quantity of extracted DNA was measured on 0.8% garose gel prepared in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0) and visualized with ethidium bromide (1.0 $\mu g ml^{-1}$) and photographed under UV light.

Amplification of the intron 1 of myostatin gene

Two polymerase chain reaction (PCR) primers, myostatin-up (5´-GAAACGGGTCATTACCATGC-3´) and Myostatin-down (5´-CATATTTCAGGCAACCAAATG-3´), targeting a fragment of 417 bp was employed in DNA amplifications as described by Hickford et al. (2009).

The PCRs were carried out in 50 µl volumes using PCR master mix kit (CinnaGen Inc., Tehran, Iran) containing 2.5 Units Taq DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 50 µM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), 0.5µM of each primer and about 100 ng of extracted DNA as template. DNA amplifications were performed using Master cycler (Eppendorf, Germany) programmed for a preliminary step of 2 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 52°C and 45 s at 72°C, with a final extension of 2 min at 72°C.

Single strand confirmation polymorphism (SSCP)

PCR products were mixed with 8 µl of denaturing loading dye (95% (w/v) deionized formamide, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue and 0.02M EDTA) in a total volume of 15 µl. The mixture was denatured at 95°C for 5 min and was snap chilled on ice (Pipalia et al., 2004).

The total volume was run in a 15% polyacrylamide gel, as described by Herring et al. (1982). The electrophoresis was performed in 0.5 × TBE buffer (Tris 100 mM, boric acid 9 mM, EDTA 1 mM) at room temperature (18°C) and constant 200 V for 3 h. Polyacrylamide gels were stained with silver according to the protocol described by Herring et al. (1982).

Statistical analysis

The allelic and genotypic frequencies, observed and expected Nei's heterozygosities (HE=1-Σ P_i^2, where P_i is the frequency of allele i) were estimated using Pop Gene32 program (ver 1.31, Canada) (Yeh et al., 1999). Hardy-Weinberg equilibrium test were calculated using Pop Gene32 (ver 1.31) program.

RESULTS

The amplification of a 417 bp fragment of the myostatin intron I gene was successful in our first attempt. All extracted DNAs from rams blood samples yielded a specific single band PCR product without any nonspecific band. Therefore, the PCR products were directly used for SSCP analysis. The allelic variation in the myostatin gene was examined by PCR-SSCP.

The non-denaturing gel electrophoresis enabled visualization of single-stranded DNA (ssDNA) and SSCP band patterns. In this study, a total of four SSCP patterns were observed in the examined sheep (Figure 1). The frequencies of the observed patterns were 0.413, 0.293, 0.130 and 0.163 for AD, AC, AE, and BC, respectively. Allele frequencies were 0.4185, 0.0815, 0.2283, 0.2065 and 0.0652 for A, B, C, D, and E, respectively (Table 1). The genotypes AD (n = 38), AC (n = 27), AE (n = 12) and BC (n = 15) occurred at a frequency of greater than 1%.

The expected homozigosity (Homexp) value for myostatin was 0.2768, expected heterozygosity (Hexp) was 0.7323 and average heterozygosity was 0.7192 (Table 2).

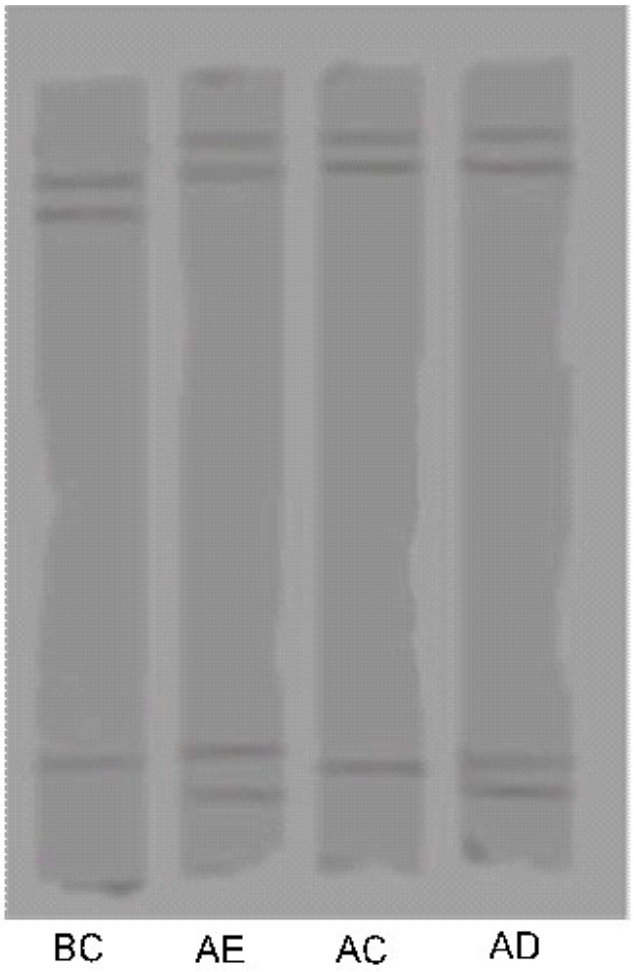

Figure 1. SSCP polymorphism of 'Makoei' sheep Myostatin gene. Four different PCR-SSCP patterns (genotype) were identified**.**

Effective number of alleles was 3.5615 and Shannon's Information index (I) was 1.4099 (Table 3). The chi-square test showed significant ($P<0.05$) deviation from Hardy-Weinberg equilibrium for this locus in studied population.

DISCUSSION

In this study, variation in the intron I sequence of the sheep ovine myostatin gene was investigated by polymerase chain reaction–single strand conformational polymorphism (PCR–SSCP) analysis. Five alleles (A, B, C, D and E) and four genotypes (AD, AC, AE and BC) were observed in myostatin intron I gene of 'Makoei' sheep. The most frequent allele and genotype in the 'Makoei' sheep breed were 0.4185 and 0.413 for allele A and genotype AD, respectively. In double muscled cattle breeds, seven different mutations in the myostatin gene namely nt821 (Del 11), nt419 (Del 7- ins 10), Q204X, E226X, C313Y, F94L and nt414(C-T)) have been identified (Kambadur et al., 1997; McPherron and Lee, 1997; Grob et al., 1997).

The effect of produced genetic variation in myostatin gene on growth and carcass traits was investigated in 517 Romney male lambs from 17 sire-lines, born on a South Island New Zealand farm. PCR-SSCP analysis of intron-1 detected five different alleles in myostatin gene. General linear mixed models revealed that the presence of allele A in a lambs genotypes was associated with decreasing in leg, loin and total yield of lean meat, whereas the presence of allele B was associated with increasing in loin yield and proportion loin yield.

Polymerase chain reaction (PCR) products of the myostatin gene amplified from sixty sheep of nine Chinese indigenous sheep breeds and one imported sheep breed were sequenced to identify the single-nucleotide polymorphisms (SNPs) in a 378-bp fragment including intron II and exon III of the myostatin gene. A total of fifteen SNPs (A1937C, T1942G, C1956T, A1972C, A1990G, A2008C, A2011G, C2019T, A2025C, A2027C, T2085G, T2173C, C2198T, C2210T and C2213T) were detected among the sixty sequenced individuals and they were all located in intron II.

Several mutations have been identified that could be responsible for different phenotypes in cattle breeds (Grobet et al., 1997; Kambadur et al., 1997; Gill et al., 2009). Mutations in exons II and III of the swine myostatin gene identified by Li et al. (2002). Zheng et al. (2008) found two SNPs located in the promoter region of the myostatin gene (T769G, C543T) and one in intron I (A1632G) in 10 goose breeds. Gu et al. (2002), using PCR–SSCP, scanned the 50 regulatory region, 30 regulatory region and a part of the coding regions of the chicken myostatin gene. They detected five SNPs (G167A, T177C, G304A, A322G, and C334T) in the 50 regulatory region and two (A6935G and A7263T) in the 30 regulatory region, in different chicken lines. They did not detect any polymorphism in the exon I region.

Results of this study, partly are in accordance with the results of Hickford et al. (2009) that five allele observed in both New Zealand and 'Makoei' sheep breeds. However, as the polymorphism is in non-coding DNA region, it is difficult to conclude how this genetic variation might be affecting myostatin activity. Possibilities include that it may affect mRNA splicing or is linked to variation elsewhere in the coding sequence that subsequently affects the amino acid sequence. 'Makoei' sheep breed is considered to be a moderating breed. In coordination with our findings, it would suggest that more research is needed to completely characterize ovine myostatin gene variation across an extended region of the gene and in a large variety of breeds from around the world.

This was the first study on the polymorphism of the myostatin locus to understand genetic variability of 'Makoei' sheep in Iran. Very little information is currently available to compare different Iranian sheep breeds. This study may be regarded as the beginning of attempts to understand the genetic variability of native sheep breeds

Table 1. Observed allele and genotypic frequencies for Myostatin locus in 'Makoei' sheep.

Allele frequency					Frequency genotypic			
A	B	C	D	E	AD	AC	AE	BC
0.4185	0.0815	0.2283	0.2065	0.0652	0.413	0.293	0.130	0.163

Table 2.Summary of heterozygosity statistics for all loci.

Locus	Obs_{Hom}	Obs_{Het}	Exp_{Hom}	Exp_{Het}	Nei	Ave_{Het}
Myostatin	0.0000	1.0000	0.2768	0.7232	0.7192	0.7192

Table 3. Summary of genetic variation statistics for all loci.

Locus	na	ne	I
Myostatin	5.0000	3.5615	1.4099

na = observed number of alleles; ne = Effective number of alleles (Kimura and Crow, 1964); I = Shannon's Information index (Lewontin, 1972).

in the Azerbaijan region. The previous breeding programs in most research centers of Iran were based on only phenotypic characters. This study confirmed the importance of molecular studies beside the morphological data in detecting genetic variation among individuals in selecting diverse parents to constructing a new population successfully.

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