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Polymorphisms in the ovine myostatin gene are associated with birth weight but not with weight gain in Iranian Makoei sheep

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ABSTRACT. Myostatin, a transforming growth factor-beta superfamily member, has been well documented as a negative regulator of muscle growth and development. Myostatin, which has 376 amino acids, is synthesized as a precursor protein. Polymorphism of the myostatin gene in Makoei sheep was investigated by PCR and single-strand conformation polymorphism technique (SSCP). Genomic DNA of 92 sheep was isolated from whole blood. A 417-bp myostatin intron I segment was amplified by standard PCR, using locus-specific primers. Four SSCP patterns, representing four different genotypes, were identified. The frequencies of the 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, respectively.

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Observed heterozygosity was 0.7192. There was significant deviation from Hardy-Weinberg equilibrium for this locus. Analysis of myostatin gene sequences revealed heterozygous SNPs, which were in agreement with results obtained in the SSCP analysis. We concluded that SSCP analysis is a quick, sensitive and reliable technique for determination of DNA polymorphisms. The effect of these genotypes on some traits was investigated, and the AD genotype was found to be associated with birth weight. No phenotypic associations were detected with the other genotypes. No associations of myostatin variants with weight gain were detected. We conclude that polymorphism in the ovine myostatin gene is associated with birth weight, but not with weight gain in Iranian Makoei sheep.

Key words: *Myostatin* gene; PCR; SSCP; SNP; Makoei sheep

INTRODUCTION

Myostatin, a transforming growth factor-beta superfamily member, negatively regulates skeletal muscle mass development by inhibiting the Myo5 and MyoD factors, which are involved in the differentiation of precursor cells into myoblasts (McPherron and Lee, 1997). Myostatin protein is first synthesized in the skeletal muscle as a 52-kDa propeptide and then prototypically processed at the conserved RSRR (arginine-serine-arginine-arginine) site to produce a 40-kDa latency-associated peptide and a 13-kDa mature peptide that form a homodimer (26 kDa) that binds to its receptor(s) to perform its biological functions (Herpin et al., 2004).

The myostatin gene consists of 3 exons and 2 introns (Bellinge et al., 2005). The effects of the myostatin gene were first described in mice, in which loss of myostatin expression in knockout mice is associated with an increase in both the number (hyperplasia) and size (hypertrophy) of muscle fibers. The muscles of myostatin knockout mice weighed about twice as much as those of wild-type mice. In the Belgian Blue and Piedmontese cattle, an extreme form of muscularity (double muscling) results from mutations in the coding region of the myostatin gene (Kambadur et al., 1997; McPherron and Lee, 1997; Wiener et al., 2009).

The ovine myostatin gene is located on chromosome 2. Single-strand conformational polymorphism (SSCP) analysis of the intron I region of this gene revealed 5 allelic variants in the New Zealand Romney sheep breed (Hickford et al., 2009). Single-nucleotide polymorphism (SNP) in the 3'-untranslated region of the myostatin gene has been shown to affect muscularity in sheep (Clop et al., 2006; Kijas et al., 2007). Additional SNPs associated with muscling have also been identified in the promoter and intron II regions (Kijas et al., 2007). In total, 23 ovine myostatin gene SNPs have been reported (Clop et al., 2006; Kijas et al., 2007; Zhou et al., 2008), and whereas 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, 4 haplotypic variants positioned at 41, +4036, and +6223 have been constructed from the coding sequence using SNPs in the myostatin gene (Kijas et al., 2007).

Quantitative trait locus studies in sheep have shown that the myostatin gene has a major effect on muscular development in Belgian Texel (Marcq et al., 2002) and muscling depth in New Zealand Romney (Hickford et al., 2009), Norwegian White (Boman et al., 2009), Cha-

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rollais (Hadjipavlou et al., 2009), and New Zealand Texel sheep (Johnson et al., 2009). The objective of present study was to characterize potential variations in the ovine myostatin gene and their association with birth weight and weight gain in Iranian Makoei sheep.

MATERIAL AND METHODS

Blood sample collection and genomic DNA extraction

Makoei sheep are fat-tailed sheep with medium bodies. They are white with black spots on the face and feet and are raised in the eastern and western Azerbaijan Provinces (Iran) mainly for meat and wool (Saadat-Noori and Siah-Mansoor, 1992). Blood samples (approximately 2-3 mL) were obtained from 92 unrelated Makoei rams coming from various parts of western Azerbaijan and stored in ethylenediaminetetraacetic acid (EDTA)-coated tubes. Genomic DNA was extracted from 0.2 mL blood using a genomic DNA purification kit (Fermentas, USA) according to manufacturer instructions. The quality and quantity of extracted DNA was measured on 0.8% agarose gel prepared in 0.5X TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA, pH 8.0), visualized with ethidium bromide (1.0 µg/mL) under ultraviolet light, and photographed.

Amplification of intron I of the myostatin gene

Two polymerase chain reaction (PCR) primers - myostatin-up (5'-GAAACGGGTCAT TACCATGC-3') and myostatin-down (5'-CATATTTCAGGCAACCAAATG-3') - targeting a fragment of 417 bp were used for DNA amplification as described by Zhou et al. (2008). The PCR was carried out in a 50-µL volume using a PCR Master Mix kit (CinnaGen Inc., Tehran, Iran) containing 2.5 U Taq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 50 µM of each dNTP (dATP: deoxyadenosine triphosphate, dCTP: deoxycytidine triphosphate, dGTP: deoxyguanosine triphosphate, and dTTP: deoxythymidine triphosphate), 0.5 µM of each primer, and 100 ng extracted DNA as a template. DNA amplifications were performed using a Mastercycler (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.

SSCP

PCR products were mixed with 8 µL denaturing loading dye [95% (w/v) deionized formamide, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue, and 0.02 M EDTA] in a total volume of 15 µL. The mixture was denatured at 95°C for 5 min and then snap-chilled on ice (Pipalia et al., 2004). The total volume was electrophoresed on 8% polyacrylamide gel, as described by Herring et al. (1982). The electrophoresis was performed in 0.5X TBE buffer at room temperature (18°C) and a constant 200 V for 3 h. Polyacrylamide gels were stained using silver nitrate according to the protocol described by Herring et al. (1982).

Sequencing

PCR products from various genotypes of the myostatin gene were subjected to DNA

sequencing. Each PCR product (10 µL) and 15 µL 5 µM myostatin-up primer were sent to CinnaGen for PCR cleanup and sequencing.

Statistical analysis

The allelic and genotypic frequencies and observed and expected Nei's heterozygosities ($H_E = 1 - \Sigma P^2_i$, where P_i is the frequency of allele i) were estimated using PopGene32 version 1.31 (Yeh et al., 1997). PopGene32 was also used to perform the Hardy-Weinberg equilibrium test.

Data on growth traits were retrieved from the national sheep recording system. The following fixed-effects model was used to calculate breeding value by using DFRIMEL (derivative-free restricted maximum likelihood; Meyer, 2000):

$$Y_{ijklm} = \mu + YR_i + SX_j + BT_k + AD_l + AN_m + E_{ijklm}$$

where Y_{ijklm} is the dependent variable (birth weight or weight gain) evaluated on the ith level of year as a random factor (YR_i, I = 1, 2, 3,…, 21), the jth level of sex as a fixed factor (SX_j, j = 1 and 2), the kth level of offspring number in each birth as a fixed factor (BT_K, K = 1, 2, and 3), the lth level of mother age as a fixed factor (AD_l, l = 1, 2,…,7), and the mth level of the random additive genetic effect (AN_m, m = number of animals for each trait). The variable μ is the overall mean for each trait, and E_{ijklm} is the random error effect. SAS was used to calculate least squares means and to make multiple comparisons among the various genotypes in the Makoei breed.

Sequence analysis

All sequences were analyzed for SNPs and mutation through direct comparison of chromatograms using SeqDoC (Crowe, 2005).

RESULTS

We successfully amplified the intron I region of the myostatin gene (a fragment of 417 bp in length) on our first attempt. All extracted DNAs from ram blood samples yielded a specific, single-band PCR product without nonspecific bands. Therefore, the PCR products were directly used for SSCP analysis.

The allelic variation in the myostatin gene was examined using a PCR-SSCP method. The non-denaturing gel electrophoresis allowed visualization of single-stranded DNA and SSCP band patterns. Four SSCP patterns were observed in the sheep (Figure 1). 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, respectively (Table 1). The observed heterozygosity value was 1.0. The chi-square test showed significant ($P \leq 0.05$) deviation from Hardy-Weinberg equilibrium for the locus under study in the Makoei sheep population.

Sequence analysis using the SeqDoC software revealed heterozygous SNPs among all examined myostatin sequences (see Figure 1). All detected SNPs were located in the same region of the gene. The results of the sequence analysis confirmed the SSCP results.

General linear mixed models revealed that genotype AD was associated with birth

weight in the Makoei sheep breed, but no association was found between the other genotypes (AC, AE, BC) and birth weight. The effect of the AD, AC, AE, and BC genotypes on weight gain was non-significant (Table 2).

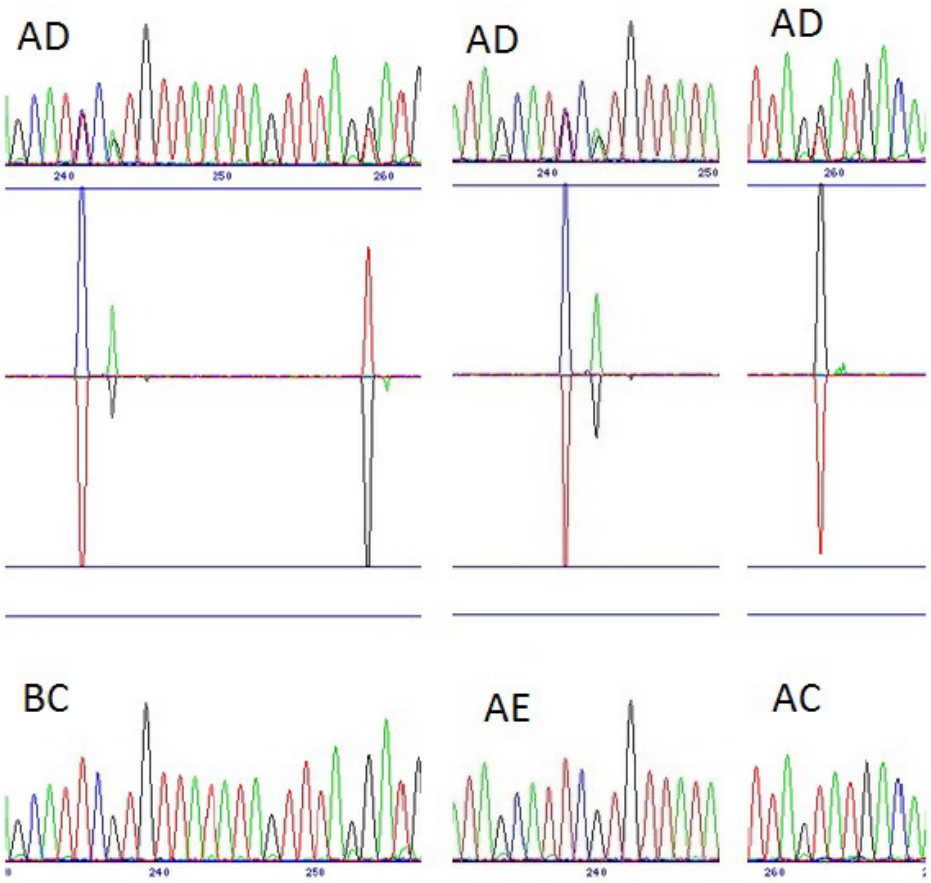

Figure1. SNPs profile of myostatin gene in Iranian "Makoei" sheep breed.

Table1. Study on genetic diversity of myostatin locus in Iranian "Makoei" sheep breed.

Genetic diversity statistics	Value	Allele frequencies	Value	Genotypic frequencies	Value
N_A	5.00	A	0.42	AD	0.41
N_E	3.56	B	0.08	AC	0.29
I	1.41	C	0.23	AE	0.13
Observed homozygosity	0.00	D	0.21	BC	0.16
Observed heterozygosity	1.00	E	0.07		
Expected homozygosity	0.28				
Expected heterozygosity	0.72				
Average heterozygosity	0.72				

N_A = observed number of alleles, N_E = effective number of alleles (Kimura and Crow, 1964); I = Shannon's information index (Lewontin and Hubby, 1971).

Table 2. Effect of the myostatin gene genotype on birth and gain weight in Iranian "Makoei" sheep breed.

P	Genotype				Trait
	BC	AE	AC	AD	
0/05	0.046 ± 0.015	0.026 ± 0.09	0.011 ± 0.0017	0.068 ± 0.020	Birth weight
NS	0.068 ± 0.178	0.084 ± 0.059	0.059 ± 0.0 92	0.12 ± 0.0570	Weight in 3 months
NS	0.163 ± 0.064	0.089 ± 0.051	0.121 ± 0.063	0.13 ± 0.037	Weight in 6 months
NS	0.18 ± 0. 057	0.145 ± 0.109	0.482 ± 0. 071	0.172 ± 0.063	Weight in 9 months
NS	0.668 ± 0.195	0.380 ± 0.301	0.598 ± 0. 02	0.88 ± 0.169	Weight in 12 months

NS = not significant.

DISCUSSION

In the present study, variation in the intron I region of the myostatin gene was analyzed in Makoei sheep using PCR-SSCP analysis. Five alleles (A, B, C, D, and E) and 4 genotypes (AD, AC, AE, and BC) were observed in the intron I region of the myostatin gene. Allele A and genotype AD were the most frequent in the sheep, with frequencies of 0.42 and 0.41%, respectively. The results of this study agree partly with those of Hickford et al. (2009), who observed 5 alleles in the myostatin gene in both New Zealand and Makoei sheep breeds.

In double-muscled cattle, 7 mutations occur in the myostatin gene - namely, nt821 (del 11), nt419 (del7-ins10), Q204X, E226X, C313Y, F94L, and nt414 (C-T) (Kambadur et al., 1997; Grobet et al., 1997; McPherron and Lee, 1997; Georges et al., 1998; Smith et al., 2000; Nishi et al., 2002). PCR products of the myostatin gene amplification in samples of 60 sheep from 9 Chinese indigenous sheep breeds and 1 imported sheep breed were sequenced to identify SNPs in a 378-bp fragment including intron II and exon III of the myostatin gene (Gong et al., 2009). Fifteen SNPs (A1937C, T1942G, C1956T, A1972C, A1990G, A2008C, A2011G, C2019T, A2025C, A2027C, T2085G, T2173C, C2198T, C2210T, and C2213T) were detected among these individuals, and they were all located in intron II.

We found that a higher number of studied animals carried alleles A and C. Only animals with genotype AD showed significantly different birth weight compared with those of the other groups (see Table 2). Among the 4 genotypes observed, 3 carried allele A (see Table 1). Significant difference in birth weight was found only in animals carrying genotype AD, so we concluded that allele D had a greater influence on the difference in birth weight. We do not believe that allele A had an influence, because we saw no significant differences between the AD and AC or the AD and AE genotypes.

None of observed genotypes in this study affected weight at 3, 6, 9, and 12 months. We expect that animals carrying the AD genotype will show a significant difference in weight gain compared with animals carrying the other genotypes. The lack of significant effect of this genotype on weight gain is likely due to inappropriate husbandry as well as the health and nutritional conditions of animals. However, because the polymorphism is in non-coding DNA, drawing conclusions about whether this genetic variation affects myostatin gene activity is difficult. It may affect mRNA splicing or be linked to variation elsewhere in the coding sequence, which subsequently affects the amino acid sequence.

Other relationships between the myostatin gene and growth have been examined. The effect of genetic variation in the myostatin gene on growth and carcass traits has been investigated in 517 male Romney lambs from 17 sire-lines born on a South Island New Zealand

farm (Hickford et al., 2009). A general linear mixed model revealed that the presence of allele A in lambs is associated with decreases in leg, loin, and total yield of lean meat, whereas the presence of allele B is associated with increases in and proportion of loin yield (Hickford et al., 2009). Several mutations in the myostatin gene may be responsible for various phenotypes in cattle breeds (Grobet et al., 1997; Kambadur et al., 1997; Gill et al., 2009). Li et al. (2002) have identified mutations in exons II and III of the swine myostatin gene. Zheng et al. (2008) have found 2 SNPs 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, have scanned the 50 regulatory region, the 30 regulatory region, and part of the coding regions of the chicken myostatin gene and detected 5 SNPs (G167A, T177C, G304A, A322G, and C334T) in the 50 regulatory region and 2 (A6935G and A7263T) in the 30 regulatory region in various chicken lines. They detected no polymorphism in the exon I region.

The work undertaken in the present study was the first attempt to examine the polymorphism of the myostatin locus to understand genetic variability of Makoei sheep in Iran. Very little information is currently available with which to compare various Iranian sheep breeds. Breeding programs in most research centers in Iran have been based solely on phenotypic characters. The current study confirmed the importance of molecular studies in addition to morphological data for the detection of genetic variation among individuals when selecting diverse parents with which to construct a new population. Additional research is needed to characterize the completely ovine myostatin gene variation across an extended region of the gene and in a large variety of sheep breeds from around the world. Of special interest are allele frequencies and the phenotypic effects of myostatin in non-meat breeds, such as merino sheep.

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