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Association of *IGF-I* Gene Polymorphisms with Carcass Traits in Iranian Mehraban Sheep Using SSCP Analysis

Research Article

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ABSTRACT

Molecular genetics selection on individual genes is a promising method to genetically improve economically important traits in livestock. The insulin like growth factor-I (*IGF-I*) gene may play important roles in growth of multiple tissues, including muscle cells, cartilage and bone. The objectives of the present study were the estimate the haplotype frequencies of the *IGF-I* gene polymorphisms in Iranian Mehraban sheep and to determine associations between polymorphisms and carcass traits in 439 individuals. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and DNA sequencing methods were employed in screening for genetic variation. Two single nucleotide polymorphisms (C114G and G116A) and two amino acid exchange (S5T and G6S) with three patterns were found in *IGF-I* gene fragment. Associations of *IGF-I* patterns with blood cholesterol and blood triglycerides were significant ($P<0.05$) and tended to be significant ($P<0.1$) for dorsal fat thick and carcass weight. The other studied traits were not significantly affected by different patterns of *IGF-I*. This finding indicates that *IGF-I* polymorphisms may be used as DNA markers for selection in the breeding process of Mehraban sheep.

KEY WORDS carcass traits, insulin-like growth factor I, Mehraban sheep, single nucleotide polymorphism, single-strand conformational polymorphism.

INTRODUCTION

The main objective of the application of molecular biology techniques to animal genetic improvement currently consists of identifying, mapping and analyzing polymorphisms of genes involved in the main metabolic pathways related to different features such as animal growth and distribution of nutrients to different tissues (Schwerin *et al.* 1995). Carcass traits are important economical traits in livestock. It has been shown that these traits are under the control of multiple genes and understanding the genetic information of related genes is helpful for the selection and breeding course through marker assisted selection (MAS) in domestic animals. Such genes can encode structural proteins or a member in a regulatory or biochemical pathway affecting the expression of the trait (Bryne and McMullen, 1996) and can be tested as putative quantitative trait loci (QTL) (Yao *et al.* 1996). The somatotrophic axis, which essentially consists of growth hormone releasing hormone (GHRH), growth hormone (GH), insulin-like growth factors I and II (*IGF-I* and *IGF-II*) and their associated binding proteins (GHBP, IGFBP1-6) and receptors (GHRHR, GHR, *IGF-IR* and *IGF-IIR*), plays a key role in the metabolism and physiology of mammalian growth (Curi *et al.* 2004). *IGF-I* is an important growth factor involved in a variety of physiological processes including cell differentiation, embryogenesis, regulation of metabolism, reproduction, fetal development, and growth (Adam *et al.* 2000; Shen *et al.* 2003). Among

the differences observed in the metabolism of animals with different body structure in the various growth phases, the role of hormones of the somatotrophic axis should be emphasized (Owens *et al.* 1993). Several lines of evidence revealed an association of the high level of *IGF-I* in the blood with fast growth in cattle (Barash *et al.* 1998; Sirotkin *et al.* 2000). In the following decades, cattle *IGF-I* gene mutations (C/T mutation in the 5'-flanking region) were also found to be associated with the percentage of fat and protein in milk (Ge *et al.* 2001; Eulalia *et al.* 2006). However, these results were quite different from Hines' discovery where no association was found between C / T mutation in 5'-flanking region and dairy production traits in Holstein cattle (Hines *et al.* 1998). The association of *IGF-I* gene polymorphism (short random repeat in the 5'-flanking region) with body weight, both at birth and weaning, has also been described (Li *et al.* 2004; Moody *et al.* 1996). However such an association was not found by Curi *et al.* (2004).

Recently an *IGF-I* intron-2 polymorphism was found to correlate positively with the twinning rate (Kim *et al.* 2009). The objectives of the present study were identification of the *IGF-I* gene polymorphisms by PCR-SSCP and DNA sequencing methods and evaluation of the association between these polymorphisms with carcass traits in Iranian Mehraban sheep.

MATERIALS AND METHODS

Sheep and DNA sources

Genomic DNA samples were obtained from 439 Mehraban rams (1 and 2 years old), which were obtained randomly from different flocks in the industrial slaughterhouse in Hamedan province, Iran. The other information including number of herds, food intake and the period of transition from the farm to the slaughterhouse were not available. Approximately 10 mL blood per sheep was collected from the jugular vein and kept in a tube containing anticoagulant ethylenediaminetetraacetic acid (EDTA). All samples were delivered back to the laboratory in an ice box. The genomic DNA was extracted from blood buffy coats of nucleated cells using a salting out procedure (Miller *et al.* 1988). The DNA samples were dissolved in TE buffer and were stored at -20 ˚C for future use.

Primers and PCR conditions

A pair of primers was designed to amplify a 265 bp fragment in exon 1 of the *IGF-I* gene (GenBank No. AY803775). These primers were as follows:

Forward: 5′-ATTACAAAGCTGCCTGCCCCTT-3′
Reverse: 5′-CACATCTGCTAATACACCTTACCCG-3′

The PCR amplification reactions were carried out in a 25 µL final volume in 0.5 mL PCR tubes and consisted of PCR buffer 10X [50 m*M* KCl, 10 m*M* Tris-HCl (pH 9.0), 0.1% Triton X-100], $MgCl_2$ (1.5 m*M*), 200 m*M* of each dNTPs, 10 p*M* of each primer, 1.5 units of Taq DNA polymerase (CinnaGen, Tehran, Iran), 50-100 ng of DNA template and distilled water. PCR method was used to optimize the reaction accuracy following: a hot start (95 ˚C for 3 min), 35 cycles were carried out (95 ˚C for 45 seconds, 60 ˚C for 40 seconds, 72 ˚C for 50 seconds), ending with a 10 min final extension at 72 ˚C. PCR products were separated by electrophoresis on 2% agarose gel (5 V/cm), stained with ethidium bromide and photographed under UV light.

SSCP and sequence analysis

Single strand conformation polymorphism (SSCP) was carried out with a Bio-Rad (Bio-Rad, USA) vertical gel. The 5 µL PCR product was diluted with 12 µL of a solution containing 98% formamide, 10 m*M* NaOH, 20 m*M* EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The mixture was denatured at 98 ˚C for 10 min, cooled in ice for 5 min and loaded on a non-denaturing 12% acry1amide: bis-acrylamide gel. Presence or absence of 10% glycerol, voltage (300 V), running time (17 h) and running temperature (4 ˚C). The gels were visualized using 0.1% silver staining.

Three samples of each pattern were selected for DNA sequencing. Primers for sequencing were the same as those used for the PCR-SSCP amplification. Gene sequences and polymorphisms were assembled and annotated using the VECTOR NTI advance 10.1.1 software (Invitrogen Corporation). Each polymorphism identified in our SNP discovery analysis was compared with the sheep NCBI dbSNP database (http://www. ncbi.nlm.nih.gov/SNP/index.html) using BLAST.

Statistical analysis

For the association studies patterns, the traits of interest including dorsal fat thickness, hot carcass weight, blood triglycerides, blood cholesterol, abdominal fat and fat tail weight were analyzed using the general linear model (GLM) procedure of the SAS program (SAS, 2004). The model for the analysis was as follows:

$$Y_{ijk} = \mu + A_i + G_j + B(W_{ijk} - W) + e_{ijk}$$

Where:
Y_{ijk}: phenotypic value of traits.
µ: overall mean.
A_i: fixed effect of the ith age (1 and 2 years old).
G_j: fixed effect of the kth pattern (3 patterns).
B: linear regression coefficient of body weight.

W_{ijk}: weight before slaughter.
W: average weight before slaughter.
e_{ijk}: random residual error.

LSMs of different patterns for traits were compared at level of 0.05 to show significant statistically difference and level of 0.1 to show significant tendency to statistically difference, respectively. Moreover the frequencies of SSCP patterns were calculated in 439 randomly selected Mehraban sheep.

RESULTS AND DISCUSSION

SSCP analysis of *IGF-I* gene

The *IGF-I* gene including 265 bp was amplified by the primers from ovine genomic DNA as expected. Three patterns of *IGF-I* gene were detected in 439 individuals. The patterns 1, 2 and 3 showed 3, 5 and 4 bands on the gel, respectively (Figure1).

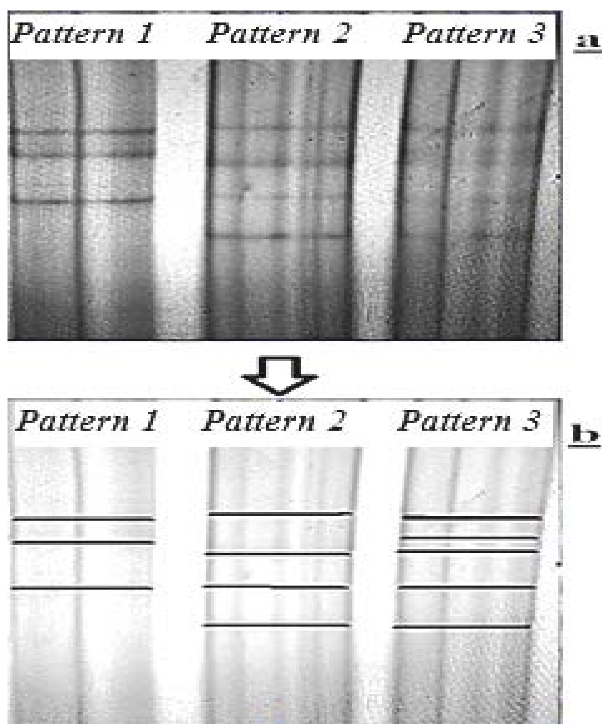

Figure 1 a. SSCP patterns of 265 bp fragment of *IGF-I* gene exon 1 of Mehraban sheep. b. Diagrammatic depiction of SSCP banding pattern of 265 bp fragment of *IGF-I* gene exon 1 of Mehraban sheep

Patterns frequencies

Patterns frequencies are shown in Table 1. The frequencies of the patterns 1, 2 and 3 were 0.08, 0.60 and 0.32, respectively. Also, the estimated allele frequencies were 0.24 and 0.76 for the A and B alleles, respectively. The patterns frequencies showed a tendency of pattern 2 > pattern 3 > pattern 1 in Mehraban flock, which had the largest frequency of pattern 2.

Table 1 Patterns frequency of SSCP variants of insulin like growth factor-I (*IGF-I*) in Mehraban sheep

IGF-I patterns	No.	Frequency
Pattern 1	35	0.08
Pattern 2	263	0.60
Pattern 3	141	0.32
Total	439	1

Sequence analysis and single nucleotide polymorphism (SNP) loci screening

Two new mutations, G→C at position 85 and G→A at position 87 located in exon 1 of *IGF-I* gene were identified by sequencing directly (Figure 2), that theses mutations deduced two amino acid exchanges S5T and G6S, respectively (Figure 3). These mutations and amino acid exchanges were not previously indicated in literature and were non-synonymous.

Statistical analysis of the *IGF-I* gene

Associations of *IGF-I* gene polymorphisms with studied traits in Mehraban sheep are given in Table 2. Pattern 1 had the largest blood triglycerides in comparison to the pattern 2 ($P<0.05$) and was tended to be significant for dorsal fat thick and carcass weight ($P<0.1$). In addition, pattern 3 does significantly blood cholesterol larger than pattern 2 ($P<0.05$). The other studied traits were not significantly affected by different Patterns of *IGF-I* gene exon 1 (Table 2). The *IGF-I* gene plays an important role in growth and function by regulating several cellular processes (Akers, 2006), including the stimulation of protein synthesis in the epithelial cells (Burgos and Cant. 2010). Furthermore, plasma *IGF-I* correlate with meat production and other studies have shown plasma *IGF-I* to be positively correlated with milk fat concentrations (Moyes, 2004). The SSCP evaluation of genes, whose product is associated with production traits, could be a valuable alternative approach for the establishment of the allelic variants useful as markers to aid selection. Apart from our own studies in sheep there is little published information on *IGF-I* polymorphisms and their association with traits of economic importance. In particular, there is a dearth of information regarding direct associations between *IGF-I* polymorphisms and carcass traits in sheep breeds. The present study describes the identification of SNPs in *IGF-I* in commercial sheep and novel associations with carcass traits (Table 2). Previous studies reporting association of variants of *IGF-I* gene with milk production and growth traits in cattle (Islam *et al.* 2009). Yilmaz *et al.* (2005) found the different patterns that corresponded with three patterns of 1, 3 and 2 in mixed breed sheep. Also, the present report shows an association of the single nucleotide polymorphism in the 5'-flanking region of the *IGF-I* gene with carcass traits in Mehraban sheep.

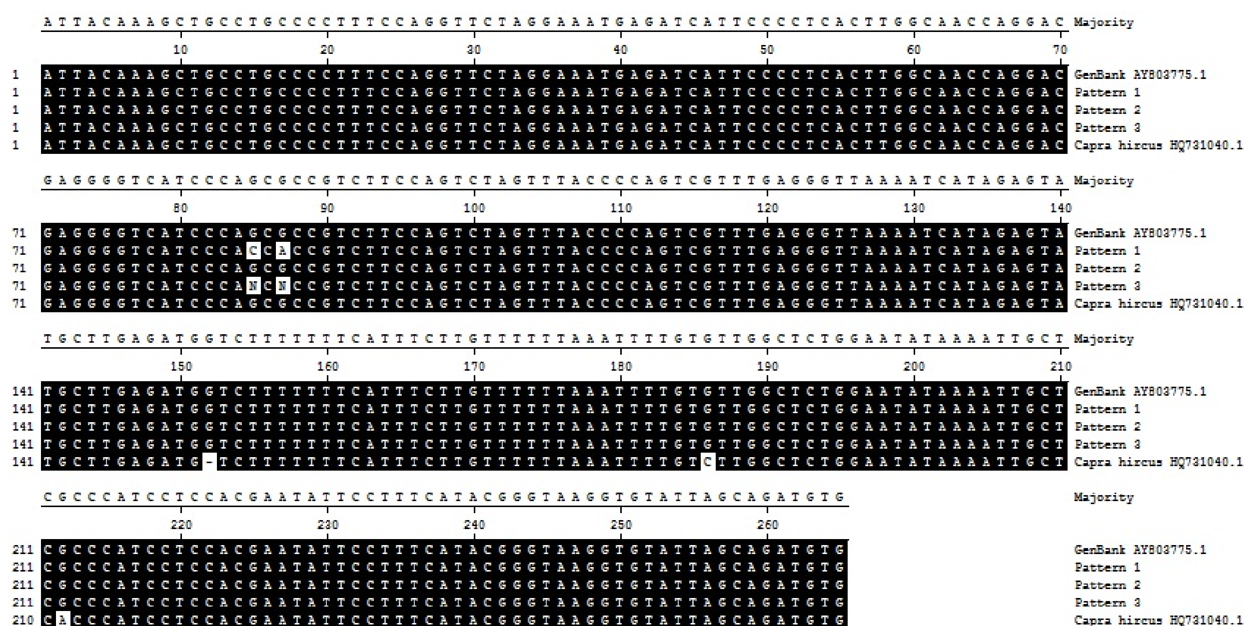

Figure 2 Comparative alignment of nucleotide sequences a 265 bp fragment of *IGF-I* gene exon 1, based on PCR-SSCP haplotype sequence in Mehraban sheep with NCBI reference sequence AY803775 and *Capra hircus*.
N: in nucleotide sequence is the heterozygote genotype

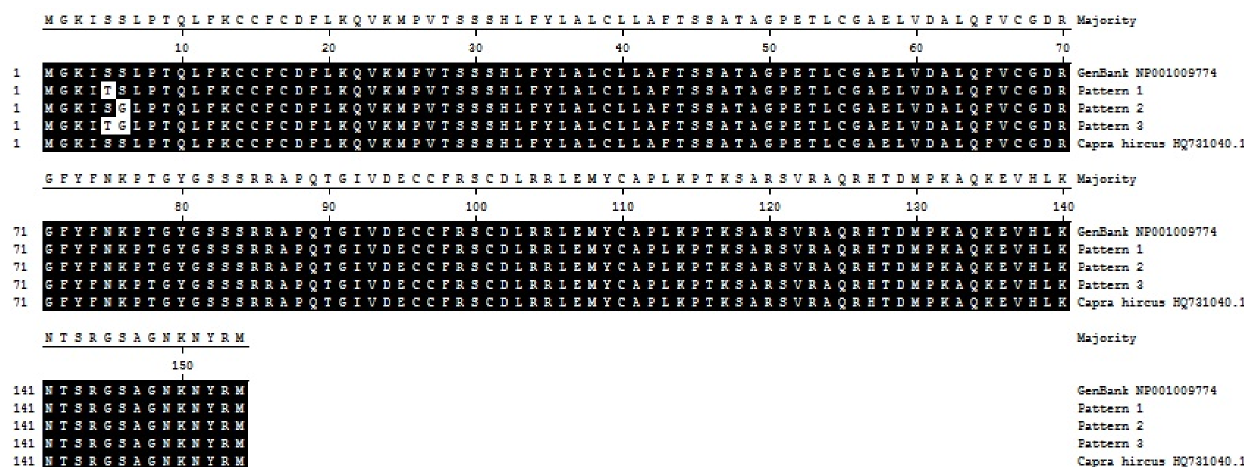

Figure 3 Comparative alignment of conceptualized full protein sequences of *IGF-I* gene exon 1, based on PCR-SSCP haplotype sequence in Mehraban sheep with NCBI reference sequence NP001009774 and *Capra hircus*

Table 2 Association of the *IGF-I* patterns with carcass traits in Mehraban sheep (Mean±SE)

Carcass traits	Pattern 1	Pattern 2	Pattern 3	P-value
Carcass weight (kg)	31.69±0.45^{a}	30.73±0.27^{b}	30.54±0.24^{b}	0.0718
Abdominal fat (kg)	1.03±0.01	1.23±0.6	1.10±0.05	0.4215
Fat-tail weight (kg)	3.93±0.38	3.64±0.22	3.95±0.22	0.3133
Dorsal fat thickness (mm)	2.31±0.47^{a}	2.08±0.28^{b}	2.22±0.26^{ab}	0.0758
Triglycerides of blood (mg/dL)	23.90±2.44^{a}	19.15±1.91^{b}	20.74±1.95^{ab}	0.0165
Cholesterol of blood (mg/dL)	58.30±3.20^{ab}	52.67±2.64^{b}	60.27±2.67^{a}	0.0178

The means within the same columns with at least one common letter, do not have significant difference (P>0.05).

IGF-I was associated with blood triglycerides and the present report shows an association of the single nucleotide polymorphism in the 5'-flanking region of the *IGF-I* gene with blood cholesterol in Mehraban sheep.

Moreover, dorsal fat thick and carcass weight were tended to be significant (P<0.1). The results of the GLM analysis of association between the *IGF-I* gene and growth traits in Mehraban sheep are summarized in Table 2 and

there was a significant difference between patterns 1 and 2. In addition the results demonstrate the highest of the heterozygous pattern 3 for blood cholesterol and of pattern 1 for blood triglycerides, dorsal fat thick and carcass weight. Ge *et al.* (2001) reported an effect of the *IGF-I* polymorphism, located in the regulatory region of the *IGF-I* gene on growth traits in Angus cattle and also suggested a direct action of this polymorphism on gene transcription and consequently on phenotypic traits. Moody *et al.* (1996), Curi *et al.* (2004), Andrade *et al.* (2008) and Casas-Carrillo *et al.* (1997) reported that a polymorphic microsatellite $(CA)_n$ located in the 5´-flanking region of the *IGF-I* gene has been associated with birth weight, weaning weight and yearling weight in different cattle breeds and with subcutaneous fat thickness in some swine families, respectively. Eulalia *et al.* (2006) reported a correlation between the polymorphism in the 5'-non coding region of the *IGF-I* and meat and milk production traits in Polish Holstein-Friesian cattle. It is well established that gene transcription is extensively and coordinately regulated. Although introns are known to carry regulatory sequences, they may not have a direct involvement in the regulation of transcription of highly expressed genes; however, systematic differences in motif distributions do suggest that introns play a role in the rate of their transcription (Zhang *et al.* 2008). Indeed, studies have described a relationship between systemic *IGF-I* and carcass fat. Davis and Simmen. (1997) reported that Angus bulls with lower plasma *IGF-I* concentrations had higher marbling scores and dorsal fat thickness. Similarly, circulating *IGF-I* was found to be negatively correlated with carcass fat percentage; fat accretion rate and fat thickness in Simmental crossbred bulls (Anderson *et al.* 1988). Furthermore, studies in transgenic mice have shown that the differentiation stage of precursor cells into mature fat cells is accompanied by enhanced expression of *IGF-I* and Rajkumar *et al.* (1999) indicating a role of *IGF-I* in fat cell developmental processes.

CONCLUSION

This finding point to a preliminary assumption that mutation of *IGF-I* gene might be used for marker assisted selection method in the Mehraban sheep. However, the researches in the future need to evaluate the mutations in other parts of this gene or other genes such as leptin and Growth hormone etc. However, the genotyping of further independent sheep populations would help to confirming the role played by this gene in meat production. It seems that evaluation of more genes in a study with more sample size and the implementation of quantitative PCR assay to measure the levels of mRNA expression of the *IGF-I* gene in the carcass of individuals with different alleles could be considered as an interesting subject for similar studies in the future.

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