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Assessment Relationship Between Leptin and Ghrelin Genes Polymorphisms and Estimated Breeding Values (EBVs) of Growth Traits in Baluchi Sheep

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Abstract: The current study was designed to estimate the frequencies of putative leptin and ghrelin genes Single Nucleotide Polymorphisms (SNPs) and investigate their association with Estimated Breeding Values (EBVs) of growth traits in Baluchi sheep. Phenotypic information about several sheep growth traits on 112 purebred Baluchi sheep was collected. Effects of the association of the variants in the third exon of the leptin gene and the variants in the first exon of the ghrelin gene on the growth traits were examined on all individuals. Three conformational patterns were identified in the leptin gene but the ghrelin gene was not polymorphic in the experimental population using PCR-SSCP (Polymerase Chain Reaction-Single Strand Conformation Polymorphism) analysis. Analysis of variance using the leptin SNP as the independent variable and EBVs as the dependent variable showed that the leptin SNP was significantly associated with additive EBVs for weight at 90 days (WW) ($p<0.05$) but no association of the leptin SNP with the other examined traits EBVs were found. The findings suggest that polymorphisms in the leptin gene might be one of the important genetic factors that influence growth traits and may be explain partial source of genetic variation.

Key words: Baluchi sheep, ghrelin gene, leptin gene, growth traits, PCR-SSCP, genetic variations

INTRODUCTION

Sheep are important meat-producing animals in Iran. Therefore, their economic values depend upon growth and productive and reproductive efficiency so the selection objective should concentrate on these traits. Producing more lambs per ewe and increasing growth performance of the lambs are two ways to increase meat production in sheep. The first objective can be achieved by increasing ewe reproductive rate including lambing rate and frequency whereas the second objective requires enhancement of the growth potential and survival of lambs.

Recently, Marker Assisted Selection (MAS) and genome analysis are being focused on by investigators and breeders. Genomic data have the potential to contribute valuable information for animal selection and are being increasingly used in the genetic evaluation of animals and design of genetic improvement programs (Banos *et al*., 2008; Dodds *et al*., 2007). The physiological regulation of feed intake, growth and energy partitioning in animals is under the control of multiple genes that regulate metabolism and energy partitioning have the potential to influence economically important traits in farm animals as do polymorphisms within these genes (Sherman *et al*., 2008). Leptin is a 16-kDa protein that is synthesized by adipose tissue and is involved in regulation of feed intake, energy balance, fertility and immune functions (Fruhbeck *et al*., 1998).

Previous studies showed that leptin is a major gene that controls feed intake and regulates body weight in mammals. Genetic differences in the leptin gene were first observed in mice (Hamann and Matthaei, 1996). Polymorphisms in the bovine leptin gene have been described and its associations with feed intake (Lagonigro *et al*., 2003; Liefers *et al*., 2002) milk production (Buchanan *et al*., 2003) and carcass and meat quality traits (Schenkel *et al*., 2005) was reported. Ghrelin is a GH releasing peptide as well as an appetite stimulant (Kojima *et al*., 1999).

Such findings suggest that variation in the leptin and ghrelin genes in domestic animals may be important in fat deposition and feed intake thereby impacting on meat quality and growth rate, respectively. However, studies about the associations of leptin and ghrelin polymorphisms with growth traits are mainly carried out in cattle but the examination of the association between SNPs and growth traits in sheep of these genes has not

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been reported. Therefore as part of the MAS program aimed at improving growth traits in Baluchi sheep we, have characterized potential variation in the ovine leptin and ghrelin genes using PCR-SSCP analysis. The objective of this study was to establish an association of genotypes with estimated breeding value of growth traits in Baluchi sheep.

MATERIALS AND METHODS

Animals and data collection: Baluchi sheep is a dual-purpose (meat and wool) breed that is fat-tailed, medium-sized (Body size varies between 35 and 40 kg in adult ewes), white-wool breed, indigenous to the southwest Pakistan, eastern Iran and southern Afghanistan, properly adapted to a wide range of harsh environmental conditions with low quality pastures. A population set of randomly selected purebred Baluchi (n = 112) lambs was screened for polymorphisms of the leptin and ghrelin genes. Data on and pedigree information for Baluchi sheep used in this study were collected at the breeding station of Baluchi sheep (in Mashhad, Khorasan Province, Iran). Initially records with implausible dates or weights were eliminated. Finally, the records of 2391 lambs having the information of pedigree (of 51 sires and 724 dams) and body weight traits were used for the analysis. The included traits were: Birth Weight (BW), Weaning Weight (WW), 6 Months Weight (6 MW), 9 Months Weight (9MW) and Yearling Weight (YW). A summary of data structure with some pedigree information for each trait is shown in Table 1.

Blood samples and DNA extraction: Blood (10 mL) was collected from each sheep by jugular venipuncture with disposable syringes into vacuum EDTA coated tubes. Genomic DNA was extracted from 100 μL of blood as described by Boom *et al*. (1990).

DNA amplification and PCR-SSCP analysis: PCR-SSCP was used to screen for polymorphism at the ovine leptin (exon 3) and ghrelin (exon 1) loci. Based on the ovine leptin gene sequence (GenBank accession No. EF534374) and the ovine ghrelin gene sequence (GenBank accession No. AY455983), two pairs of oligonucleotide primers were designed to amplify two fragments using the primer premier 5.0 software (http://www.primerbiosoft.com/biosoft.com) (Table 2).

PCR amplification was accomplish in a final volume of 25 mL containing 75 mM Tris-HCl (pH 8.8), 1 unit of Platinum Taq DNA Polymerase (Invitrogen, USA), 0.1 MG ML^{-1} BSA (Roche, Mannhein, Germany), 0.2 mM each of dNTPs (Pharmacia, Uppsala, Sweden), 2 mM $MgCl_2$, 25 pMol of primers and 100 ng of DNA template. Amplification was performed in a thermal cycler T-Personal (Biometra, Germany) with the following program after an initial denaturation step at 94°C for 10 min, 35 cycles were programmed as follows: 94°C for 30 sec, 66.5°C (leptin) or 59°C (ghrelin) for 30 sec, 72°C for 30 sec and final extension at 72°C for 7 min.

The DNA fragments were visualized on an agarose gel by ethidium bromide staining and exposure to ultraviolet light. Each lamb was genotyped by using PCR-SSCP. PCR products was diluted in denaturing solution (95% of formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue), denatured at 95°C for 5 min, chilled on ice and amplicons were subject to SSCP analysis to screen for polymorphisms using 12% polyacrylamide gels at 350 V and 6°C for 7 (leptin) and 4 h (ghrelin) in 1×TBE buffer. The gels were stained with silver.

Estimated breeding values: Breeding values for growth traits (Birth Weight (BW), Weaning Weight (WW, 90 days of age), weight at 6 months (W 6), weight at 9 months (W 9) and Yearling Weight (YW)) were estimated for all of 2391 animals using the Best Linear Unbiased Prediction (BLUP) based on an animal model with a relationship matrix. To identify fixed effects to be included in the models, least square analysis were conducted using the General Linear Model (GLM) procedure. This was performed on a model including fixed effects (year-5 classes; herd-2 classes; age of dam in years-8 classes; sex-2 classes and type of birth-3 classes). All of these fixed effects were significant ($p<0.05$) for all of the traits and were then included in the models. For the analysis in which records were not adjusted for age of

Table 1: Characteristics of the data sets

No. of samples	Growth traits				
	BW	WW	6MW	9MW	YW
Records	1854.00	1725.0	1325.00	1262.00	1154.00
Animals[a]	2251.00	2251.0	2251.00	2251.00	2251.00
Sires[b]	45.00	45.0	45.00	44.00	44.00
Dams[b]	560.00	549.0	519.00	503.00	481.00
Grand-sires	32.00	32.0	31.00	29.00	29.00
Grand-dams	141.00	137.0	130.00	125.00	115.00
mean	4.18	21.2	31.79	33.79	40.54
CV (%)	14.15	15.0	14.26	12.81	11.10

[a]Animals in pedigrees. [b]Numbers of sires or dams with progeny with records

Table 2: Sequence and position of oligonucleotide primers used for the leptin and ghrelin genes

Gene	Location	Length	Primer	Sequence (5-3)
Leptin	Exon 3	275 bp	Forward	gctccaccctctcctgagtttgtcc
			Reverse	tgtcctgtagagacccctgtagccg
Ghrelin	Exon 1	112 bp	Forward	cctgctctggatggacttggc
			Reverse	ggcttggggcatttaggacg

lamb this effect was taken into account by fitting as a linear covariate. Estimation of variance and covariance components was obtained by Restricted Maximum Likelihood (REML) using a Derivative-Free (DF) algorithm (Meyer, 1989), fitting an animal model. Maternal genetic or permanent environmental effects were taken into account by including appropriate random effects in the model (Meyer, 1997). Convergence was assumed when the variance of likelihood values in the simplex was <10-8. In addition, a restart of each analysis was performed with different starting values to attempt to avoid convergence to local maxima. The general representation of the animal model used is as follow:

$$Y = Xb+Zu+Wm+Spe+e$$

Where:

- Y = The a n×1, vector of records
- b = A vector of fixed effects in the model with association matrix
- X, u = The vector of direct genetic effects with association matrix
- Z, m = The vector of maternal genetic effects with association matrix
- W, pe = The vector of maternal permanent environmental effects with association matrix
- S and e = The vector of residual (temporary environment) effects

The variance-covariance structure for the model is as follow:

$$V\begin{pmatrix} u \\ m \\ pe \\ e \end{pmatrix} = \begin{pmatrix} A\sigma_u^2 & A\sigma_{um} & 0 & 0 \\ A\sigma_{mu} & A\sigma_m^2 & 0 & 0 \\ 0 & 0 & Ic\sigma_m^2 & 0 \\ 0 & 0 & 0 & Ie\sigma_e^2 \end{pmatrix}$$

Where:

- A = The numerator relationship matrix
- Ic = The an identity matrix with order number of ewe
- Ie = An identity matrix with order number of records
- σ_u^2 = The direct genetic variance
- σ_m^2 = Maternal genetic variance
- σ_{um} = Covariance between direct and maternal genetic effects
- σ_{pe}^2 = Variance due to maternal permanent environmental effects
- σ_e^2 = The variance due to residual (temporary environmental) effects

Association analysis: For the association studies, the traits of interest were analyzed using the GLM procedure of the SAS program and least squares means of the genotypes were compared by the Tukey test. The linear model used was:

$$Y_{ij} = \mu+G_i+e_{ij}$$

Where:

- Y_{ij} = The breeding value for each trait of the ijth animal
- G_i = The fixed effect of the ith class of genotype
- e_{ij} = The random error effect associated with the ijth observation

Means were compared using the Tukey test in the SAS program. Differences with $p<0.05$ were considered statistically significant. Maternal and animal estimated breeding values were independently compared between animals that have different genotype on the leptin and ghrelin genes by Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

An understanding of influencing factors and genetic principles affecting the growth traits is needed to implement optimal breeding and selection programs. Use of DNA markers to account for genetic variation for quantitative traits provides producers a tool to assist in genetic selection of superior animals. Previous studies were shown that polymorphism on growth traits candidate genes might be one of the important genetic factors that influence growth traits and maybe explain partial source of genetic variation on Baluchi sheep (Tahmoorespur *et al*., 2009; Taheri *et al*., 2009).

Genetic polymorphism of the leptin and ghrelin genes in Baluchi sheep: The leptin and ghrelin genes were chosen because they have been shown to be involved in the regulation of appetite, metabolism and growth (Chilliard *et al*., 2005). Previous studies showed that variation of these genes affected gene expression at the transcription or translation levels certainly affected economic traits significantly. The desirable fragment of the leptin (275 bp fragment of exon3) and ghrelin (112-bp fragment of exon1) genes were amplified from ovine genomic DNA as expected. PCR-SSCP method was used to identify the polymorphism in exon3 and exon1 of the ovine leptin and ghrelin genes, respectively. Screening for polymorphisms in the two analyzed fragments revealed polymorphic site in the leptin gene while the 112-bp region of ovine ghrelin locus didn't show polymorphism in Baluchi sheep. Until now several Single Nucleotide Polymorphisms (SNPs) have been identified in the bovine leptin gene. These include four SNPs located

Table 3: Least square means and standard errors of the growth traits of Baluchi sheep according to the SNP genotype in Leptin

		Growth traits				
Locus	Pattern (frequency)	BW	WW	6 MW	9 MW	YW
Leptin	L1 (n = 28)	4.60±0.13	25.42^{a}±0.72	36.18±0.95	32.11±1.09	41.91±1.33
	L2 (n = 63)	4.42±0.08	23.52^{ab}±0.52	33.78±0.62	34.62±0.71	43.12±0.87
	L3 (n = 21)	4.30±0.15	22.23^{b}±0.93	33.49±1.13	34.28±1.29	43.15±1.57
	p-value	0.34	0.03*	0.08	0.15	0.72

Means of sheep average daily gain with different superscript letters (a and b) were significantly different (Tukey test, *p<0.05)

Table 4: Parameter estimated of analyzed traits

Traits	σ_a^2	σ_m^2	σ_e^2	σ_g^2	h^2	m^2
Birth Weight (BW)	0.05	0.07	0.23	0.35	0.14	0.200
Weaning Weight (WW)	0.22	0.79	9.10	10.13	0.02	0.070
Weight at 6 months (W 6)	0.58	1.60	18.36	20.55	0.02	0.070
Weight at 9 months (W 9)	0.16	1.12	17.47	18.75	0.08	0.050
Yearling Weight (YW)	2.30	0.01	17.88	20.28	0.11	0.008

at exon 2 (Buchanan *et al*., 2002; Haegeman *et al*., 2000) and located leptin promoter region (Nkrumah *et al*., 2005) whereas only Zhou *et al*. (2009) recently found four Single Nucleotide Polymorphisms (SNPs) in ovine leptin gene. Sherman *et al*. (2008) found the A/G SNP in bovine ghrelin gene. In spite of the importance of these genes in regulation of body weight and feed intake in mammalian until now there have been no study conducted on their association with sheep growth traits, therefore single nucleotide polymorphisms within these genes were examined for effects on these traits in Baluchi sheep. The SSCP patterns (genotypes L1, L2 and L3) frequencies of the leptin gene are shown in Table 3. SSCP pattern (genotype) L2 is the predominant type in the set of analyzed Baluchi sheep (63 out of 112 Baluchi sheep with records = 56.25%). The remaining genotype frequencies are 25% (L1) and 18.75% (L3).

Parameter estimation: Genetic parameters were estimated with a univariate animal model including the direct additive genetic effects, maternal additive genetic effects and maternal permanent environmental effects under Restricted Maximum Likelihood (REML) procedures (Table 4). Appropriate model for BW and WW should included direct additive genetic effects as well as maternal permanent environmental effects. However, the most appropriated models for 6MW, 9MW and YW had only the direct additive genetic effects. We calculated the means of five important growth traits (BW, WW, 6 MW, 9 MW and YW) with SAS 9 by the set up model (Table 3). Type of birth, sex, age of dam and year of birth had significant influences on body weight traits ($p<0.05$). Also, the age of lamb at weaning time had a significant influence on 6 MW, 9 MW and YW.

Low direct heritability estimation for all traits except birth weight found in the present study may be due to the low nutritional level, poor quality of the pasture at the sheep breeding station and harsh climate conditions especially in this stage of growth period suggest that this environment is not favorable for expression of genetic potential of Baluchi lambs although low estimates of direct and maternal heritabilities for growth traits may be indicate that selection for growth traits will result in slow genetic improvement. Also in the study, the maternal heritability for growth traits were estimated was nearly the direct heritability indicating the importance of maternal additive genetic effects on the traits of concern. Maternal heritability with the corresponding values of ram for body weights decline with increasing age from birth to adult and the maternal genetic effect scarcely affect the post-weaning or late growth which theory is also supported by Safari *et al*. (2005). For pre-weaning growth traits, both the genetic and permanent environmental effects of dam are very important contributions to the phenotypic values. Therefore, genetic evaluation of pre-weaning growth traits needs to adopt a model that includes direct and maternal genetic as well as permanent maternal environmental effects. Low additive genetic and high residual variances in WW, 6 W, 9 MW and YW could be explained by the harsh environmental conditions of the range coincided with these ages. The genotype frequencies of SNP in leptin showed a trend toward association with weaning weight.

Genetic effects on growth traits: The results showed that the SSCP pattern (genotype) of leptin is associated with breeding values of growth traits (Table 5). It observed a significant effect of this polymorphism on WW yield ($p<0.05$). The L1 and L3 genotype has the lowest and the highest additive estimated breeding value for the birth and weaning weight, respectively. The L3 genotype was associated with the highest and the lowest additive estimated breeding value for birth and weaning weight (pre-weaning traits), respectively. On the other hand, although, L3 animals presented the lowest EBV for WW and the highest for BW, the highest WW seemed to be related with the lowest BW, causing the lowest EBV for

Table 5: Least square means and standard errors of the estimated breeding value (values denote deviations from the mean of traits) of growth traits of Baluchi sheep according to the SNP genotype in leptin

		Weight estimated breeding values (means±SE, kg)						
		BW		WW				
Locus	Pattern (frequency)	Additive	Maternal	Additive	Maternal	6 MW (additive)	9 MW (additive)	YW (additive)
Leptin	L1 (n = 28)	0.03±0.02	-0.013±0.01	0.05^{ab}±0.02	-0.13±0.05	0.04±0.05	0.05±0.01	0.35±0.11
	L2 (n = 63)	0.04±0.01	0.025±0.01	0.05^{a}±0.01	-0.10±0.03	0.05±0.03	0.07±0.01	0.17±0.07
	L3 (n = 21)	0.05±0.02	0.009±0.02	-0.02^{b}±0.02	-0.20±0.06	0.15±0.05	0.06±002	0.21±0.13
	p-value	0.82	0.085	0.046*	0.37	0.29	0.77	0.40

Means of sheep average daily gain with different superscript letters (a and b) were significantly different (Tukey test, *p<0.05)

BW. Similar associations were observed for L1 genotype. Any association with other growth traits was not observed. The observed relationships for post-weaning traits can be related to increased body weight of L3 animals (Table 4). The L1 genotype (leptin) was associated with YW in Baluchi sheep.

Results of the preliminary analysis of variance are shown in Table 3. The effects of genotypes on five main growth traits in Baluchi sheep population were all insignificant except for WW. In the tested Baluchi sheep population at the birth the mean body weights of L1 genotype (4.60 kg) were 0.18 and 0.3 kg higher than those of L2 (4.42 kg) and L3 SSCP pattern (4.30 kg), respectively. This difference was found to be not significant (p>0.05). Body weight at weaning (90 days) was still considerably (p<0.05) higher in L1 lamb in comparison with the other two groups in fact L1 lamb's WW was 25.42 kg while WW in L2 and L3 animals were 23.52 and 22.23 kg, respectively. No significant differences in body weight were found comparing different genotype either at the age of 180 days (6MW) at the age of 270 days (9 MW) and the age of 360 days (YW). Moreover, there was a tendency that L2 genotype individuals had better performance in post weaning traits such as 6 MW, 9 MW and YW than other genotype although no significant differences appeared (p>0.05). In addition, L1 genotype animals have better performance in pre-weaning growth traits in contrast other genotype but these animals have worse performance in post-weaning traits even though no statistical differences (p>0.05) presented. A significant effect of leptin locus was found only on additive breeding value for weaning weight (Table 4). A significant difference was detected between genotypes L2 and L3 but the average breeding values of all observed parameters were the highest in animals with L1 genotype. L3 genotype was associated with the highest breeding value for birth weight and lowest breeding value for weaning weight while the highest breeding values for post-weaning traits were associated with genotype L1. The lowest mean of breeding values for all traits were associated with genotype L3. The result showed no significant association for leptin on post weaning weight that may be environmental factors are likely to play a large role on post weaning weight and failing to control this factor could confound results leading to non-significant findings. The estimated effects suggest that most of the impact of leptin is realized prior to weaning. Because maternal effects were larger in early stages of the life we only assessment relationship between maternal effect in pre-weaning traits and leptin gene polymorphism. The result showed that animals with L3 genotype have highest breeding value for BW while these animals have lowest breeding value for maternal effect in WW it may be consequence of pleiotropic effect of genes that control two traits also it may be occur as a result of fat deposit in ewes organs such as mammary glands that possibly reduce maternal ability.

CONCLUSION

In this study, the first association between a SNP in the ovine leptin gene and growth traits in Baluchi sheep is characterized. The optimized PCR-SSCP conditions identified the various alleles accurately, rapidly and inexpensively compared to other methods which have been used to type genes. Summarizing the effects of the leptin gene polymorphism on the studied traits, the following conclusion could be drawn: The L1 genotype appeared favorable for several meat production-related traits but L3 genotype appeared lowest favorable several meat production-related traits. Animals with the (L2) genotype at the exon3 appeared superior in maternal trait.

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