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Association of Growth Traits with SSCP Polymorphisms at the Growth Hormone Receptor (GHR) and Growth Hormone Releasing Hormone Receptor (GHRHR) Genes in the Baluchi Sheep

Mehdi Vafaye Valeh, Mojtaba Tahmoorespour, Mazyar Ansari, Mohammad Reza Nassiry, Davood Karimi and Amir Taheri
Department of Animal Science, Faculty of Agriculture,
Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, I.R. Iran

Abstract: The study of candidate genes, based on physiological effects, is an important tool to identify genes to be used in marker-assisted selection programs. On the basis of sheep genome mapping information the ovine GHR and GHRHR genes was examined as a possible genetic marker for growth traits in sheep. Evaluation of the genetic diversity for 2 genes in 102 animals of the Iranian indigenous sheep breed (Ovis Aries) Baluchi was done. The genotypes for GHR and GHRHR loci were determined by the PCR-SSCP method. Single-nucleotide Polymorphisms (SNP) (Exon10) were discovered in the Growth Hormone Receptor (GHR) gene but Growth Hormone Releasing Hormone Receptor (GHRH-R) was monomorphic. Moreover, associations were studied between the polymorphism at GH Receptor (GHR) loci and growth trait in the growing Baluchi sheep. The traits analyzed were Birth Weight (BW), Average daily Gain from Birth to Weaning (GBW), weaning to 6 month (GWS) and from 6 month to Yearling Age (GSY). In the association studies, traits of interest were analyzed using the General Linear Model (GLM) procedure of the SAS program and least squares means of the genotypes were compared by the Tukey test. Only significant associations ($p<0.05$) were observed between the genotype of the GHR polymorphism and Average daily Gain from Birth to Weaning (GBW). These results confirmed the potential usefulness of GHR gene in marker-assisted selection programs for sheep breeding.

Key words: GHR, GHRHR, polymorphism, baluchi sheep, growth traits, PCR, SSCP

INTRODUCTION

The efficiency of sheep production enterprises can be improved by enhancing litter size, lamb weight, milk yield and wool quantity and quality. In Iran, demand for meat product has increased in the last 2 decades and will continue to rise due to the high rate of population growth. Lamb and mutton are traditional sources of protein and the consumption levels are high in comparison with meat from beef cattle and goats. The sale of lamb provides a considerable economic return in most Iranian breeds of sheep and accounts for 30-40% of the agriculture output value. To increase economic returns from these animals, there needs to be improvement in the weight and in the growth rate of lambs, so the selection objective should concentrate on this trait (Tosh and Kemp, 1994). The sheep population in Iran is composed mainly of fat tailed, carpet-wool native breeds (Farid *et al*., 1977).

Baluchi sheep is the major common native breed and account for about 29% of total sheep population. Most genetic studies on the growth of sheep have concentrated on birth weight, weaning weight and yearling weight (Bathaei and Leroy, 1998) these traits are the evaluations of growth over a relevant economic time period. Substantial advances have been made over the past decades through the application of molecular genetics in the identification of loci and chromosomal regions that contain loci that affect traits of importance in livestock production (Andersson, 2001). This has enabled opportunities to enhance genetic improvement programs in livestock by direct selection on genes or genomic regions that affect economic traits through marker-assisted selection and gene introgression (Dekkers and Hospital, 2002). In farm animals, promising candidate genes for many traits are in the Growth Hormone (GH) axis. The GH (Growth Hormone) gene pathway contains various interdependent genes, such as GH, IGF1 (Insulin-like Growth Factor), PIT1 (Pituitary Specific Transcription Factor), GHRH (Growth Hormone Releasing Hormone), somatostatin GHRHR (Growth Hormone Releasing

Corresponding Author: Mehdi Vafaye Valeh, Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, I.R. Iran

Hormone Receptor), GHR (Growth Hormone Receptor) and others (Cogan and Phillips, 1998). The Growth Hormone Receptor (GHR), a cell-surface mediator of actions of growth hormone, is a member of the cytokine/ hematopoietin superfamily of receptors. Only a few reports are published on the effects of GHR gene polymorphism on growth and meat production traits in farm animal. Hale *et al*. (2000) have shown a correlation between the lengths of the variable TG-repeat in the P1 promoter of the bovine GHR gene and growth rates in young Angus cattle; the (TG) 11 GHR allele was associated with lower growth rates in Angus steers. These results were confirmed by Curi *et al*. (2005) and Maj *et al*. (2005) found a marked effect of genetic variants of the bovine GHR gene 5/-noncoding region on meat production-related traits.

Growth Hormone (GH)-Releasing Factor (GRF, GHRH) is the hypothalamic peptide that specifically stimulates both synthesis and secretion of pituitary GH. After reaching the pituitary, GRF binds to its specific receptors on somatotrophs and generates bursts of GH secretion episodically (Frohman *et al*., 1992). Mutations in the coding region of the GHRHR are present in approximately 10% of patients with familial Isolated GH Deficiency (IGHD) type IB, characterized by autosomal recessive transmission, low but measurable serum GH levels and a favorable response to GH therapy (Salvatori *et al*., 2001; Connor and Kappes, 1999) found that differences among beef bulls in their GH response to intravenous injection of GHRH are related to differences in growth performance and carcass composition and believe that these differences are mediated at the GHRH-R and that GHRH-R may be an important candidate gene controlling growth performance and carcass traits of beef cattle.

In spite of the functional importance of GHRHR and GHR in the regulation of GH, there was no association an analysis with polymorphism has been reported in sheep. The aim of this study is the evaluation of genetic variability of Growth Hormone Releasing Hormone Receptor (GHRHR) and Growth Hormone Receptor (GHR) genes using a nonradioactive SSCP protocol and to investigate the relationship between these polymorphisms and growth traits of Baluchi sheep. This intends to be a first step for a deeper study of Baluchi breed in order to establish a breeding program based on marker-assisted selection.

MATERIALS AND METHODS

Animals: One-hundred and two purebred Baluchi sheep, progeny of 18 rams, were randomly selected from 2 separate research flocks of Baluchi sheep kept at the Abbasabad Sheep Breeding Station, located northeast of Mashhad, Iran. The number of half-sibs varied from 1-16. The data set and pedigree information used in this research were pre and post weaning body weights (Birth Weight (BW), Weaning Weight (WW), 6-Month Weight (6MW) and Yearling Weight (YW)) gathered from 2000-2003 on Baluchi sheep From Breeding Station of Baluchi sheep. Lambs were weighed and ear-tagged at birth time. Weaning was at approximately 3 months of age. All lambs were weaned at the same day, without necessarily at the same age. Moreover Lambs were weighed monthly at the same day. The structures of data set used in this research are presented in Table 1.

DNA extraction: Blood samples (10 mL) were obtained by jugular venipuncture, using vacuum tubes treated with 0.25% Ethylenediaminetetracetic Acid (EDTA). DNA was extracted from 100 μL of blood as described by Boom *et al*. (1990). After measuring DNA concentration and its purity by spectrophotometry, DNA was diluted to a final concentration of 50 ng μL^{-1} in water and stored at 4°C.

DNA amplification by PCR: Twenty five microliter of Polymerase Chain Reaction (PCR) mixture were carried out in 0.5 mL PCR tubes, using a PCR kit (GenePack PCR Universal) with the lyophilized components. Each tube contained 1.5 units of Taq DNA polymerase, 10 mM of Tris-HCl (pH 9), 50 mM of Kcl, 1.5 mM of $MgCl_2$ and 200 mM of each dNTP. To this mixture, 1 μL of each primer (50 ng μL^{-1}), 22 μL of water, 1 μL of DNA (50 ng μL^{-1}) and 2 drops of mineral oil were added. Based on the ovine GHR gene sequence (GenBank accession No. AY292283) and the ovine GHRHR gene sequence (GenBank accession No. AY292289), 2 pairs of oligonucleotide primers were designed to amplify 2 fragments using the primer premier 5.0 software (http://www.primerbiosoft.com/biosoft.com) (Table 2).

Amplification were carried out for GHRHR in a biometra T-personal ver: A 1.11 thermocycler following a hot start (95°C for 3 min), 35 cycles were carried out (95°C for 45 sec, 62°C for 40 sec, 72°C for 50 sec), ending with a 10 min final extension at 72°C. For growth hormone receptor gene a different annealing temperature (60°C) was used. Amplification was verified by electrophoresis

Table 1: Description of data set

Item	BW	WW	6MW	YW
No. record	102.00	102.00	102.00	97.00
Mean (kg)	4.40	24.97	36.09	44.07
S.D (kg)	0.70	4.55	5.03	5.34
C.V	16.11	18.23	13.94	12.12

BW = Birth Weight; WW = Weaning Weight; 6MW = 6-Month Weight; YW = Yearling Weight

Table 2: Sequence and position of oligonucleotide primers used in the Growth Hormone Receptor (GHR) and Growth Hormone Releasing Hormone Receptor (GHRHR) genes

Gene	Size of PCR prod	Location	Primer number	Primer sequence
GHR	218 bp	Exon 10	1	5′-GCCAAAACAATAAGACTGGGAACC-3′
			2	5′-GGCTGTAGTGGTAAGGCTTTCTGTG-3′
GHRHR	255 bp	Intron 10	3	5′-ATTGTGGGCGATGAGTGAGGAG-3′
			4	5′-AGCGGAATGAGGAGAAGCGTTGA-3′

on 1.5% (w v^{-1}) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris-Borate, pH 8.3), using a 50 bp ladder as a molecular weight marker for confirmation of the length of the PCR products. Gels were stained with ethidium bromide (1 μg mL^{-1}).

Single-strand conformation polymorphism analysis: PCR products were resolved by SSCP analysis. Several factors were tested for each fragment in order to optimize the methodology: amount of PCR product (4-15 μL), dilution in dena-turing solution (20-85%), denaturing solution (A: 95% of formamide, 1 0 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue; B: same as A, plus 20 mM of EDTA), acrylamide concentration (6-14%), percentage of crosslinking (1.5 and 5% C), presence (10%) or absence of glycerol, voltage (100-350 V), running time (2-12 h) and running temperature (4, 6, 10 and 15°C). Each PCR reaction was diluted in denaturing solution, denatured at 95°C for 5 min, chilled on ice and resolved on nondenaturing polyacrylamide gel. The electrophoresis was carried in a vertical unit (Paya pajoohesh VEU-7350, 160×140×0.75 mm), in 1× TBE buffer. The gels were stained with silver.

Statistical analysis: Prior to statistical analyses, Weaning Weight (WW), 6-Month Weight (6MW) and Yearling Weight (YW) traits were adjusted by age of lamb. Average Daily Gain (ADG) was calculated as the difference between the initial and final body weights divided by the number of days of the test. Assumptions of normality of data distribution and equality of variances within sire and genetic categorizations were tested. In the present study, the structure of data was very close to paternal half sib group design. The best approach would than be to analyze the data within sire. A sire was required to have at least 2 progeny to be included in these analyses. The effects of genotypes on the traits studied were analyzed by the least-squares method as applied in the General Linear Model (GLM) procedure of JMP 7.0 (SAS Institute Inc., 2007) according to the following statistical model:

$$Y_{ijkqrls} = \mu + G_i + S_j + T_k + b_1(B_{ijkqrls} - \overline{B}) + b_2(A_{ijkqlrs} - \overline{A}) + Sr_q + D_r(Sr_q) + Y_l + e_{ijkqrls}$$

where:

Y_{ijkqr} = Studied traits
μ = The overall mean
G_i = The fixed effect of GHR genotypes (i = 1, ...3)
S_j = The fixed effect of sex (1 = male, 2 = female)
T_k = The fixed effect of birth type (1 = single; 2 = twin)
b_1, b_2 = The linear regression coefficient of trait on birth weight and age of dam, respectively
Sr_q = Fixed effect of sire (1, ... 18)
$D_r(Sr_q)$ = Dam nested within sire as random effect
Y_L = The fixed effect of year (1, 2, 3)
e_{ijkqrs} = The random residual error, assumed normally and independently distributed

Least squares means of the genotypes were compared by the Tukey test. Significance was determined as $p<0.05$, unless otherwise specified. For Birth Weight (BW), we included age of dam as a covariate, while for Weaning Weight (WW) and average daily Gain from Birth to Weaning (GBW), the covariate considered was the birth weight and age of dam.

The non-significant effects and corresponding interactions were discarded for the final analysis. In order to avoid unreliable results, genotypes with very low frequency (<0.05) in the total animal sample were not included in the analysis. Of the 102 animals, 3 were excluded of the statistical approach due to missing values or unreliable data.

RESULTS

DNA amplification by PCR: Figure 1 shows a typical result of a PCR amplification of the two analyzed fragments. Based on the sequence of the GHR gene in sheep, primers 1 and 2 allowed the amplification of a fragment of 218 bp from nucleotide 53-270 (Genebank AY292283) that corresponds to exon10 of this gene and Primers 3 and 4 were designed for the amplification of a fragment of 255 bp, from nucleotide 229-483 in sheep (Intron10 of the GHRHR gene, Genebank AY292289) (Table 2).

Single strand conformation polymorphism analysis: After optimization of the parameters that affect the detection of SSCPs, we analyzed the PCR products from 102 animals, with the conditions described in Table 3. Electrophoresis was carried out at 250 V and 7°C in gels

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

		Growth trait				
		Pre-weaning		Post-weaning		
Locus	Pattern (frequencies)	BW (kg)	WW (kg)	GBW (g day^{-1})	GWS (g day^{-1})	GSY (g day^{-1})
GHR (Exon10)	A/A (32)	4.35±0.13	24.60	209±5^{b}	116±60	44±80
	A/G (62)	4.49±0.10	24.96	214±5^{a,b}	119±50	34±70
	G/G (8)	4.11±0.22	25.82	224±6^{a}	108±13	48±17
	p-value	ns	ns	0.046*	ns	ns

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

Table 4: Conditions of electrophoresis in SSCP analysis

Gene	Acrylamide (%)	DNA (µL)	Denaturing solution (µL)	Temp. (°C)	Duration (h)
GHR (Exon10)	8	15	17	4	4
GHRHR (Inton10)	12	8	12	7	6

Table 5: Means and standard deviations of analyzed traits

Traits	Mean±SD	CV
Birth Weight (BW) (kg)	4.40±0.70	16.11
Weaning Weight (WW) (kg)	24.97±4.55	18.23
Average daily Gain from Birth to Weaning (GBW) (g day^{-1})	215±40	19.20
Average daily Gain from Weaning to 6 month Weight (GWS) (g day^{-1})	116±33	28.44
Average daily Gain from 6 month Weight to Yearling Weight (GSY) (g day^{-1})	44±19	44.44

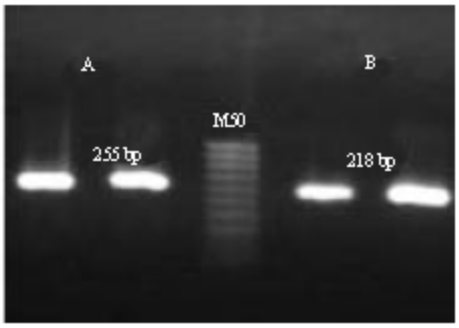

Fig. 1: PCR products analysed by electrophoresis in a 1.5% agarose gel with ethidium bromide stairing. A: GHRHR (Intron 10); B: GHR (exon 10)

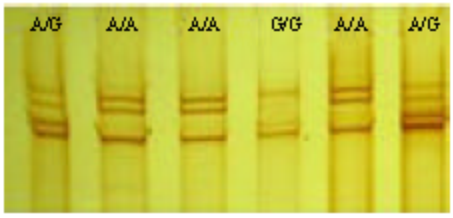

Fig. 2: The PCR-Single-Strand Conformation Polymorphism (SSCP)-based DNA test for Growth Hormone Receptor (GHR) exon 10 polymorphism

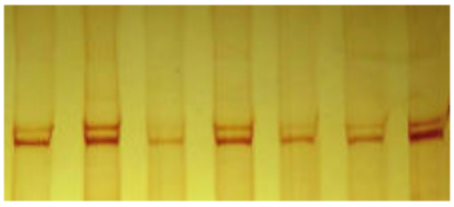

Fig. 3: The PCR-Single-Strand Conformation Polymorphism (SSCP)-based DNA test for Growth Hormone Releasing Hormone Receptor (GHRHR) intron 10 polymorphism

without glycerol, for exon10 of the GHR gene and for intron10 GHRHR, electrophoresis was carried out at 280 V and 4°C and the gel contained 2% of glycerol. Figure 2 shows the SSCP band pattern for growth hormone receptor gene exon-10. We obtained three different conformational patterns. The frequencies were 61% for pattern 1 (A/G), 31.2% for pattern 2 (A/A) and 7.8% for pattern 3 (G/G) (Table 3). For the 255 bp fragment of Intron10 of the GHRHR gene, the best results were obtained under the conditions described in Table 4. Separation of single strands was clear and in this case, animals were monomorphic (Fig. 3).

Animal performance: The means for all traits analyzed and standard deviations are given in Table 5.

Association of genotypes with growth traits: The establishment of relationships between genotypes and growth traits was done in 102 samples. Frequencies of GHR genotypes in the analyzed populations and the least squares means and standard errors for respective traits are presented in Table 5. Significant statistical results were found in daily Gain from Birth to Weaning (GBW) between individuals with genotype A/A and G/G ($p<0.05$). Individuals with genotype G/G of GHR gene had a superior daily Gain from Birth to Weaning (GBW) and a lighter birth weight when compared to those of

individuals with other genotype ($p>0.05$). However, no significant association of different genotypes with other traits was detected ($p>0.05$). All fitted effects were significant for body weight before 3 months of age (pre-weaning period). Age of dam had a significant effect on the pre-weaning growth period but declined quickly after weaning (90 days). GWS and GSY were influenced significantly by sire, sex and year of birth ($p<0.05$) but not by age of dam and birth weight ($p>0.05$). Type of birth effect influenced early weight changes but had no significant effect on post-weaning traits ($p>0.05$).

DISCUSSION

Growth is a composite of complex developments that are influenced by genetic, nutritional and environmental factors. Pre-weaning (BW, WW, GBW) and Post-weaning (GWS, GSY) growth traits have an important impact on the profitability in the sheep industry. Therefore, breeding for optimal BW and larger gains is a major consideration in sheep breeding programs. Mapping of QTL and identification of causative genes that affect growth traits will greatly enhance the progress towards this goal. Hormones, growth factors and other regulatory proteins associated with so called somatotropic axis are candidate markers for quantitative traits in farm animals. Till now, >10 variants of the farm animal GHR and GHRHR have been detected (Connor and Kappes, 1999; Sun *et al*., 1997; Polasik *et al*., 2005; Viitala *et al*., 2006; Maj *et al*., 2005; Ge *et al*., 2003; Hui-Fang *et al*., 2008; Curi *et al*., 2005; Hale *et al*., 2000; Falaki *et al*., 1996) but most of them focused on the milk production traits (Falaki *et al*., 1996; Blott *et al*., 2003; Jiang and Luci, 2001), growth traits (Curi *et al*., 2005; Hale *et al*., 2000; Maj *et al*., 2005; Ge *et al*., 2003; Aggrey *et al*., 1999) and some studies described only the polymorphism, not the association analyses (Connor and Kappes, 1999; Sun *et al*., 1997; Polasik *et al*., 2005; Horikawa and Bruce, 2001). Whereas this study are mainly carried out in the cattle.

In spite of this, no reports are published on the effects of GHR and GHRHR genes polymorphism on growth traits in sheep. This study was designed as the first step in detecting genetic markers for growth traits of baluchi sheep, which would eventually provide useful information for the MAS program. In the present study, we assessed the association between different GHR and GHRHR genotypes and growth traits including the Body Weight at birth (BW) and Weaning (WW), pre-weaning average daily gain (GBW) and post-weaning average daily gain (GWS, GSY) of baluchi sheep. The effects of different genotypes were estimated.

In our study, GHRH-R intron 10 was monomorphic Fig. 3; while Pariset *et al*. (2006) found SNP (AY292289:g. 339G.T) in 8 European sheep breedsby Taqman method. Connor and Kappes (1999) found Eco57I RFLP in bovine GHRH-R and have shown associations between the polymorphism at the Eco57I site in the intron 6 of the GHRH-R gene and growth performance and carcass traits of beef cattle. Horikawa and Bruce (2001) cloned the ovine and bovine pituitary GHRH receptors (GHRHRs). The ovine receptor (oGHRHR) encodes a protein that is similar to that of porcine, human, rat and mouse with, respectively, 84.3, 80.7, 75.9 and 74.0% amino acid identity. Surprisingly, oGHRHR has a 16 amino acid truncation at its carboxyl-terminal end when compared with GHRHRs from other known mammals. In humans, Mutations in the coding region of the GHRHR gene have been reported in patients with familial Isolated GH Deficiency (IGHD) typeIB (Salvatori *et al*., 2001).The absence of diversity does not imply that genes are not polymorphic. It only means that the primers used do not delimitate a polymorphic region.

For GHR exon 10, we observed three conformational patterns in Baluchi sheep Fig. 2, Pariset *et al*. (2006) analysed the same exon with the Taqman methodology and found a SNP (AY292283:g. 122A.G) in the 8 European ovine breeds. In bovine breeds, GHR polymorphisms are extensively studied and some associations between production traits and polymorphism have been made. Hale *et al*. (2000) have shown a correlation between variable Gt repeat in the P1 promoter of the bovine GH receptor gene and growth rates in young Angus cattle; the TG11 GH receptor allele is associated with lower growth rates in Angus steers. Falaki *et al*. (1996) reported on the effect of RFLP-TaqI in the GHR gene 3' end on breeding value for milk protein in Italian Holstein-Friesian cattle. Aggrey *et al*. (1999) have shown associations between the polymorphism at the AluI site in the 5'-noncoding region of the GHR gene and milk production traits. Holstein bulls with the AluI (+/+) genotype of the GHR gene had a higher breeding value for milk fat than bulls with the (-/-) genotype. Ge *et al*. (2000), found 4Single Nucleotide Polymorphisms (SNP) in exon10 of the bovine growth hormone receptor gene. Di Stasio *et al*. (2005) have shown associations between the polymorphism at the AluI site in the exon10 of the GHR gene and meat production and quality. In the tested baluchi sheep population, sheep with G/G genotype weighed about 1.2 kg heavier than those with A/A genotype at weaning ($p>0.05$). Significant statistical results were found in average daily Gain from Birth to Weaning (GBW) between individuals with genotype A/A and G/G ($p<0.05$). Individuals with genotype G/G of GHR

gene had a superior daily Gain from Birth to Weaning (GBW) and a lighter birth weight when compared to those of individuals with other genotype ($p>0.05$). Moreover, there was a tendency that G/G genotype individuals had better performance in other aspects such as weaning weight and growth rate than AA genotype although, no significant differences appeared ($p>0.05$). Besides, A/G genotype animals measured about 1 kg lighter than those with G/G genotype at weaning and a heavier birth weight when compared to those of individuals with other genotype ($p>0.05$) and the tendency that A/G genotype animals had worse performance in other aspects existed although no statistic differences ($p>0.05$) presented (Table 5). However, these results should be interpreted with precaution due to the small sample size.

Also our data show that PCR-SSCP is a simple and efficient technique for the detection of single base substitutions and can be employed for evaluating genetic variability in large populations. The identified gene variants, however, need large population studies in order to establish a breeding program for marker assisted selection/improvement in productivity of the sheep resources of Iran.

CONCLUSION

We can conclude that PCR-SSCP in GHR is a promising new possibility to select for increased GBW through selection for the G/G genotype. However, it is necessary to confirm our findings on a larger number of animals and with another breed before definitive conclusions can be made.

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