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Association of the polymorphism in the 5' flanking region of the ovine IGF-I gene with growth and development traits in Makuisheepof Iran

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ABSTRACT

The insulin-like growth factor 1 (IGF-I) gene has been described in several studies as a candidate gene for growth.Thispreliminary study attempts to identify associations between growth traits and genetic polymorphisms at the 5' flanking region IGF-I in Makui sheep. DNA samples from100Makuisheep,anindigenous Iranian breed,wereevaluated in the research. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of the 5' flanking region (Exon1) of the ovine revealed that the IGF-I gene had the following three banding patterns (genotypes); A/A, A/G and G/G. The evaluation of an association between these SSCP patterns with birth weight (BW), weaning weight (WW),six month weight(SW),nine month weight (9W), average daily gain from birth to weaning (GBW), weaning to six months (GWS), from six months to nine months (GSN), from nine months to yearling weight(GNY) and developmenttraits in oneyear demonstrated a positive effect of the pattern (Genotype) A/G with GBW and weaning weight (WW)andsix month weight(SW). The pattern (Genotype) A/Ademonstrated superior birth weight when compared to those with other gene patterns.GBW, GNY and biometric or developmenttraits were influenced significantly by sex ($p<0.01$)exceptRL: rump length trait.Also type of birth effect influenced early weight changes but had no significant effect on GSN, GNY traits($p>0.05$).These results demonstrated that IGF-I gene could be a genetic locus, or linked to a major gene that significantly affects growth and the afore-mentioned economic traits in sheep.

Key words: Makui Sheep, IGF-I gene, growth and Biometric orDevelopment traits, PCR-SSCP

INTRODUCTION

Makui is a breed of sheep classified as fat-tailed, similar to Turkish White Karamanandrepresentsanimportant multi-purpose sheep for production in the East and WestAzerbaijan provinces of Iran. Its population is estimated at about 2.7 million [1]. The breed is well adapted to cold and highland environments [2]. They are multi colored: black, white withblack spots on face and feet [3]. Many indigenous sheep including the Makuibreed face many challenges includingfrequent drought, disease, conflictand poor nutrition,they are consequently at risk of extinction. As these sheephave adapted to harsh environmental conditions they have undergone natural selection as defense against these conditions. Consequently, the native breeds have become an important resourcefor poorfarmers and herdsmen. Quantitative traits in these sheep are often controlled by many genes. Localization of QTL's (Quantitative Trait Loci) can be done by linkage disequilibrium analysis or by a candidate gene approach. Candidate genes have known biological functions related to the development or physiology of an important trait [4].The Insulin-Like Growth Factors (IGFs) play a major role in regulating cell proliferetion and inhibiting apoptosis. IGFs are expressed

ubiguitously and act as an autocrin/paracrine manner through binding to the IGF-I reseptor (IGF-IR). The bioactivity of IGF in tissues is determined by both local and systemic factors. The local factors included the levels of thereceptors that are expressed, various IGF binding protein(IGFBPs) and IGFBP protease.The mature IGF-I and IGF-II peptides consist of B and A chain of insulin. Unlike insulin, the IGF peptides are not prototypically cleaved, but remain linked in the mature peptides by C domains analogous to the C peptide of insulin. Buth IGF-I and IGF-II contain an additional short D domain that is not found in insulin. The IGF-I and IGF-II Prohormones contain a C-terminal E peptide that is cleaved in the Golgi apparatus during secretion[5].

Insulin-like growth factors one and two (somatomedins- IGF-I and IGF-2) are structurallyrelated proteins thathave a key role in cell differentiation, embryogenesis, growth and regulation ofmetabolism [6]. Due to their role in regulation of cell proliferation and animal growth, the IGF-I and its gene are considered as candidate markers for growth rate and meat production traits in mammalian.TheIGF-1 gene that is located on chromosome 3 in sheep is a marker for growth rate and meat production and has an important role in mammary gland cell differentiation and proliferatio[7]. In humans, pigs, goats, rats, and chickens, the IGF1 nucleotide sequence is about 70-90 kb [8].Exon numbersdifferbetween species; for example, goats, pigs and sheep have 1-6 exons [9](Mikawa et al., 1995), and humans and rats 1-5 [10].[11]in a comparative study of the IGF-IA precursor between human and bovine species, identified a conservation of around 93 and 96% in nucleotide and amino acid sequences, respectively. Body height, body length, chest width and rump length are four main growth traits that significantly impact on the production of a sheep's meat. Therefore, breeding for optimal growth traits and higher gains are the main considerations in goat and sheep breeding programs. Most genetic variation is represented by single nucleotide polymorphisms and many of them are believed to cause phenotypic differences between individuals. Identification of causative mutations that affect growth traits will greatly enhance progress towards this goal.

The objectives of this study were to search for the same polymorphism in the 5'flanking region of IGF-I in Iranian Makui sheep that was found by [12]Yilmaz et al., (1998) in purebred Polypays and crossbreeds consisting of the Hampshire, Targhee, Rambioullet, Dorset and Suffolk breeds using a non-radioactive SSCP protocol and to investigate the relationship between thesepolymorphisms and growth traits of Makui sheep. This is intended to be the first step of a more in-depth study of the Makui breed in order to establish abreeding program based on marker-assisted selection.

MATERIALS AND METHODS

The analysis was done on a sample of 100 Makui sheep owned by the ResearchCentre for Animal Breeding and Selection, Maku, Western Azerbaijan. Bloodsamples were collected into a 5 ml EDTA contained vacutainer tube and transferred within 2 hours to thelaboratory for DNA extraction.Total DNA extractions were made with a modified salting out method [13](Miller et al., 1998) from whole fresh blood. The DNA samples were quantified usingBiophotometer (Eppendorf) and stored in −20°C in aliquots. 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)), it was collected from 2000 to 2003 from sheep at the Breeding Station of Makui. Lambs were weighed and ear-tagged at birth. All lambs were weaned on the same day, though not necessarily at the same age and lambs were weighed monthly on the same day.The 265-bp fragment of the 5' flanking region of the ovine IGF-I gene was amplified. Based on the ovine IGF-I gene sequence (GenBank accession no. AY803775)marker genotype determination and blood IGF-I was evaluated. Genomic DNA was extracted from whole blood using the salting out method [13](Miller et al., 1988). The IGF-I genotypes were identified with the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique. PCR was used to amplify the 265-bp DNA fragments from genomic DNA. PCR contained 25-50 ng genomic DNA, 10 pmoL of each primer, 2 µL 10X PCR buffer, 1.5 mM MgCl2, 200 µMdNTP and 1 unit Taq-polymerase, in a total volume of 20 µL. Sequences of the primers that were used in PCR had previously been reported by Yilmaz et al., (1998)[12]and(Ge et al., 2001)[25]. Sequences of IGFR and IGFF were'5'-CACATCTGCTAATACACCTTACCCG-3' and 5'-ATTACAAAGCTGCCTGCCCC-3', respectively. Conditions for PCR were 95°C for 2 min, followed by 31 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 30s. The final step was at 72°C for 4min. For analysis of Single-Strand Conformation Polymorphism (SSCP) several factors were tested to optimize the methodology: Amount of PCR product (4 – 15 µL), dilution in denaturing solution (20 - 85%), denaturing solution (A: 95% of formamide, 10 mMNaOH, 0.05% xylene-cyanol and 0.05% bromophenol blue; B: same as A, plus 20 mM of EDTA), acrylamide concentration (6 - 14%), percentage of cross linking (1.5 to 5%), 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 a denaturing solution, denatured at 95 °C for 5 min, chilled on ice and resolved on non-denaturing polyacrylamide gel. The electrophoresis was carried in a vertical unit (Payapajoohesh VEU-7350, 160×140×0.75 mm), in 1× TBE buffer. The gels were stained with silver.

Prior to statistical analyses, birth weight (BW), weaning weight (WW), 6-month weight (6MW), and yearling weight (YW) traits were adjusted according to the age of lamb. Average daily gain (ADG) was calculated as the difference between the initial and final body weights and divided by the number of days in the test.

Statistical analysis

To determine associations, the traits of interest were analyzed using the general linear model (GLM) procedure of the SAS program [14](Statistical Analysis System, 1997), according to the following statistical model:

$$Y_{ijklm} = \mu + G_i + S_j + ls_k + B(W_{ijkl} - \overline{W}\ldots) + SR_l + G_i \times LS_k + e_{ijklm}$$

Where:

Y_{ijklm}= phenotypic value of trait, μ = the overall mean, G_i= the fixed effect of IGF-I genotypes (i = 1..., 3), S_j= = the fixed effect of sex (j = 1, 2), ls_k= the fixed effect of litter size(k=1,2), B= the linear regression coefficient of trait on birth weight, SR_q= fixed effect of sire, G_i.LS=the interactionbetweengenotypeand litter size,e_{ijklm}=the random residual error.

RESULTS

To determine allelic and genotypic frequencies of the IGF1 polymorphism100 sheep were genotyped for polymorphism in the IGF-I gene. The PCR amplified a 265bp fragment from exon1 of the ovine IGF-I gene. After optimization of the parameters that affect the detection of SSCP's, the PCR products from 100 animals were analyzed. The PCR-SSCP analysis of exon1 IGF-I revealed three distinct patterns (Figure 1)

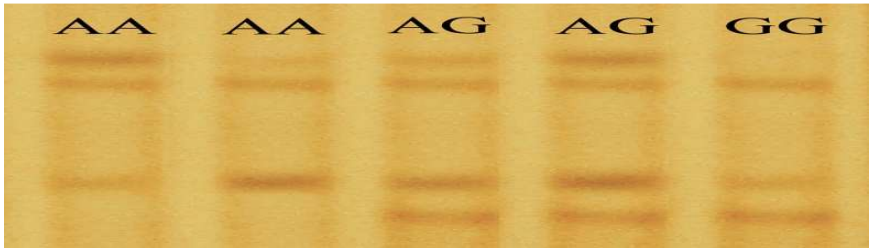

Figure 1.SSCP polymorphism of 'Makui' sheep IGF-1gene. Three different PCR-SSCP patterns (genotype) were identified.

[15]Yilmaz et al., (2005) found that the same patterns corresponded with the three genotypes A/A, A/G, and G/G in mixed breed sheep. The frequencies were 52 for pattern (A/A), 42 for pattern (A/G) and 6 for pattern (G/G) .

Table 1. Least square means (± s.e) of the growth traits of Makui sheep according to the SNP genotype in IGF-I

	Growth traits				
IGF-1	**BW**	**W3**	**W6**	**W9**	**W12**
AA	3.91±0.08^{a}	18.94±0.40^{b}	27.94±0.72^{b}	28.36±0.67	35.78±1.14
AG	3.82±0.09^{ab}	19.61±0.55^{b}	29.08±0.82^{b}	28.98±0.75	35.92±1.26
GG	3.42±0.17^{b}	16.11±1.07^{a}	23.47±1.5^{a}	27.97±1.52	35.36±2.45
P value	3.5*	4.57*	5.34**	0.39^{ns}	0.02^{ns}

	Growth traits			
IGF-1	**GBW**	**GWS**	**GSN**	**GNY**
AA	0.177±0.007^{b}	0.094±0.007	0.004±0.011 ^{a}	0.083±0.011
AG	0.202±0.009^{a}	0.098±0.009	-0.010±0.02 ^{a}	0.094±0.012
GG	0.159±0.012^{b}	0.088±0.013	0.042±0.019 ^{b}	0.110±0.018
P value	6.36**	0.19^{ns}	2.32^{ns}	0.87^{ns}

Within columns means with different superscript, (a, b) were significantly different (Tukey test). GBW, GWS, GSN, GNY (k**g/day**). BW-WW-SW (**Kg**).

In the tested Makui sheep population, significant statistical results were found inaverage daily gain from birth to weaning (GBW) between individuals withthe genotype AG andthe other genotypes. Individuals with the genotype AGof IGF-I gene had a superior average daily gain from birth to weaning (GBW) when compared to those individuals with other genotypes. Individuals with genotype GG of IGF-I gene had the leastWW and SW when compared to those individuals with other genotypes.In the tested Makui sheep population, significant statistical results were found in BW between individuals with genotypes AA and GG.The effect of the IGF-I gene was significant for average daily gain from birth to weaning (GBW), birth weight (BW), weaning weight (WW), six month weight(SW) and body length(BL)(Table 1,2).

Table 2. Least square means (± s.e) of the biometric orDevelopment Traits of Makui sheep according to the SNP genotype in IGF-I

	Growth traits					
IGF-1	**BL**	**CH**	**HW**	**HB**	**SC**	**RL**
AA	56.19±0.94^{a}	83.35±1.0	67.11±0.93	66.33±1.17	8.15±1.18	34.18±0.85
AG	57.35±1.18^{a}	83.84±1.3	66.70±1.16	63.66±1.46	8.35±1.48	33.91±1.07
GG	61.22±1.86^{b}	86.12±2.1	70.91±1.84	67.76±2.31	8.24±2.33	33.62±1.69
P value	3.18*	0.74	2.34	1.88	0.01	0.44

BH:body height, WH: wither height, BL: body length, CH: chest width, RL: rump length

GBW,GNY, and biometric traits were influenced significantly by sexexceptfor the trait of rump length (RL)(Tables 1 and 2).Type of birth effect influenced early weight change but had no significant effect on post-weaning traitsGSN,GNY(Table 1).Sire had a significant effect on GBW,CH traitsand G_i.LS had a significant effecton GBW. The ANOVA test summary for growth and biometric orDevelopment Traits of Makui sheep were shown at table 3 &4.

Table 3. ANOVA test summary for growth Traits of Makui sheep

source	**df**	**BW**	**WW**	**SW**	**GBW**	**GWS**	**GSN**	**GNY**
Genotype	2	3.5*	4.57*	5.34**	6.36**	0.19	2.32	0.87
Sex	1	-	-	-	12.38***	5.43	0.31	59.32***
Ls	1	54.68***	39.12***	9.69**	7.25**	0.24*	0.06	0.08
Bw	1	-	-	-	4.75*	1.16	2.21	0.33
Sire	13	-	-	-	1.86*	1.56	0.89	1.60
G.LS	2	-	-	-	3.57*	0.49	0.47	0.72
R-Square	-	0.43	0.38	0.21	0.59	0.32	0.24	0.56
Cv	-	10.17	12.36	13.09	12.7	29.18	1067.86	42.52

Table 4. ANOVA test summary for biometric orDevelopment Traits of Makui sheep

source	**df**		**BH**	**WH**	**BL**	**CH**	**SC**	**RL**
Genotype	2		1.88	2.34	3.18*	0.74	0.01	0.44
Sex	1		14.18***	21.03***	18***	43.10***	241.24***	2.83
Ls	1		0.53	1.26	0.16	1.27	1.23	0.22
Bw	1		1.26	2.11	5.42*	2.67	0.43	0.99
Sire	13		1.15	1.07	1.69	2.49*	1.06	0.65
G.LS	2		0.68	0.21	1.11	1.43	1.09	0.07
R-Square	-		0.39	0.48	0.44	0.59	0.83	0.22
Cv	-		6.43	4.97	5.96	4.61	51.73	8.87

BH:body height, WH: wither height, BL: body length, CH: chest width, RL: rump length, SC (Testis girth)

DISCUSSION

Substantial advances have been made over the past decades from the application of molecular genetics for identification of loci and chromosomal regions that contain loci affecting important traits importance in livestock production [16].This has enabled opportunities to enhance genetic improvement programs in livestock by direct selection of genes or genomic regions that affect economic traits through marker-assisted selection and gene introgression [17].The IGFs signaling system, which composed of IGF-I, IGF-II, IGF-I receptor, IGF-II receptor and six binding proteins (IGFBP-1~IGFBP-6), play an important role in development, growth and reproduction as well as ageing[18].The critical survival pathway activated by IGF-I stems from IRS-I. IRS-I recruits and stimulates the

PI-3 kinase (PI-3K), which then transmits signal to the serine/threonine kinase Akt. The activated Akt phosphorylates and block a variety of propoptopic proteins, including BAD, caspase-9, beta kinase. Furthermore, Akt induces the expression of antiapoptopic proteins, for example, Bcl-2. Other mitogenic survival of IGF-IR pathways involves signal transducers and activator of transcription (STATs) that are phosphorylated and activated by IGF-I through JAK-1/2 and PI-3K/AKt pathway[5](Vipin et al., 2007).The absence of line differences in plasma IGF-I levels between GL and FC lines in spite of clear difference in growth rate in plasma GH concentrations may support that plasma IGF-I does not appear to be GH-dependent implying the importance of other factors besides GH in the regulation of IGF-I in chickens. Male and female broiler chickens selected for 6-week body weight (GL) line or for feed efficiency between 3 and 6 week of age (FC line) were used.[19].

Ge et al., (2001)[24] reported an effect of the IGF-I/SnaBI polymorphism, located in the regulatory region of the IGF-I gene, on growth traits in Angus cattle and suggested that polymorphism had a direct action on gene transcription and consequently, on phenotypic traits. A nucleotide transversion from G to C was identified at intron 4 of the IGF-I gene in Nanjinag Huang goats. Two alleles and three genotypes were observed in this group. The results showed that polymorphism of the IGF-I gene was associated with BW, W6, W12, G2, L6, H6, H12 and G12 ($p<0.05$). The goats with the CC genotype had significantly higher BW, W6, W12, G2, L6, H6, H12 and G12 than those with the GC genotype, and had significantly higher W12, H6, H12 and G12 than those with the GG genotype[20].The least square means of the 3genotypic classes of the IGF-I-P2 locus in Chinese Beef Cattle showed that there were not significant associations between IGF-I-P2 and all biometric traits(BW: body weight; HW: height at withers; BL: body length; HG: heart girth; RW: rump width)that corresponded with these result in Makui sheep [21].

In the Nanjiang Cashmere Goat population, IGF-1-P1 loci, the polymorphism of IGF-1 gene is significantly associated with cashmere production traits. The cashmere fineness of AA genotype individual was significantly lower than that of AB genotype ($p<0.05$). The body weight of AC genotype individual was significantly higher than that of BB genotype ($p<0.05$), [18].The effect of bGH genotypes on the estimated breeding value for milk related traits by [22](Sadeghi et al.,2008) reported that the differences in the milk and fat yield between the two genotypes approached significance ($p<0.01$). Bull with LL genotype had higher milk, fat yield compared to LV genotypes for the GH Locus.

The SSCP analysis of genes, whose product is associated with production traits, could be a valuable alternative approach to establish the allelic variants that would be useful as markers to aid selection. In the present study associations were searched between a SNP (single nucleotide polymorphism), in the 5' flanking region of the ovine IGF-I gene and growth traits in Makui sheep.For a fragment of 265bp in the 5' flanking region of the ovine IGF-I gene, 467 to 732bp upstream from the 5' end of Exon1, three conformational patterns were observed. Yilmaz et al., (2005)[15].found the same patterns that corresponded with the three genotypes A/A, A/B, and B/B in mixed breed sheep. This report has also demonstrated an association of the single nucleotide polymorphism in the 5'-flanking region of the IGF-I gene with growth traits inMakuisheep. The results of the GLM analysis of associations between the IGF-I gene and growth traits in Makui sheep are summarized in Table 1 &2. Results of this study, are partly in accordance with the results of [23]Tahmoorespur et al.,(2009), that reported thatthe effect of the IGF-I gene was significant ($P <0.05$) for average daily gain from birth to weaning (GBW),while the effect of the IGF-I gene was significant ($P <0.05$) for average daily gain from birth to weaning (GBW), birth weight (BW), weaning weight (WW), six month weight (SW)in Makui sheep. Individuals with the GG genotype of IGF-I gene had lowerWW and SW when compared to those of individuals with other genotypes ($P <0.05$). In addition the results demonstrated superiority of the heterozygous A/G genotype for weaning weight, and of the A/A genotypefor birth weight.To date, this was the second study that attempted to detect allele variation in the ovine IGF-I gene and its association with growth traits in Iranian breeds of sheep**.** To make the selection schemes applicable, further analysis of the effects ofIGF-Ipolymorphisms, using populations from differentgenetic backgrounds and increasing the size of samples sets is recommended. However, the small size of the data set makes it difficult to perform a true assessment and these results are to be confirmed through studies with a larger dataset.

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