IJAS Iranian Journal of Applied Animal Science

Molecular and Bioinformatics Analysis of Allelic Diversity in *IGFBP2* Gene Promoter in Indigenous Makuee and Lori-Bakhtiari Sheep Breeds

Research Article

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Received on: 5 May 2018
Revised on: 7 Jul 2018
Accepted on: 31 Jul 2018
Online Published on: Jun 2019

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Online version is available on: www.ijas.ir

ABSTRACT

The aim of this study was to perform molecular and bioinformatics analysis of *IGFBP2* gene promoter in association with some economic traits in indigenous Makuee (MS) and Lori-Bakhtiari (LB) breeds. DNA was extracted from blood samples of 120 MS and 200 LB and a 297 bp fragment from the upstream sequences of studied gene was amplified and genotyped by single-strand conformational polymorphism (SSCP) technique. Two genotypes of AB and BB were seen in MS and LB breeds. Then one sample from each genotype was send to sequencing. After obtaining the sequencing result, the sequences homology was performed on the National Center for Biological Information NCBI server by basic local alignment search tool (BLAST) program. The alignment of the obtained sequences and their comparison with reference sequences from the Gene Bank were done using CLUSTALW multiple alignment tool of BioEdit software. In addition, the DNASIS MAX software was used to identify DNA motifs. The bioinformatics analysis revealed differences in sequences of *IGFBP2* between observed genotypes. Ten motifs in promoter sequence of *IGFBP2* genes were seen, so that the CAP_site motif was most abundant in both fragments motif. Statistical analysis using general linear method model (GLM) procedure of SAS software showed significant ($P<0.05$) association of *IGFBP2* with thigh round (TR) trait in Makuee sheep. Further studies in other indigenous sheep breeds and investigation of other genetic regions along with regulatory sites seem to be necessary.

KEY WORDS *IGFBP* gene, Lori-Bakhtiari sheep, Makuee sheep, promoter, sequencing.

INTRODUCTION

Given the important role of sheep in providing the main part of meat consumption in the country and high diversity of breeds in Iran as well as the use of wool of this animal in the carpet industry, studying the genes and economic traits in sheep is particularly interested in animal breeding programs. In Iran, the sheep meat is more consistent with Iranian people's tastes and temperament than beef. However, the amount of meat produced inside the country is not meeting the needs of a growing community; therefore, to increase its production must take the necessary steps to breeding the native sheep breeds. The Makuee sheep (MS) is a dual purpose (meat and wool) breed and its main habitat is in Marand and its surroundings in northwest of Iran (Karimzadeh *et al.* 2016). This breed is superior to other indigenous breeds because of high efficiency of meat production and the availability of high quality white wool. Four to five year old rams reach 140 kg (live weight) and their carcass weight is up to 75 kg, and male lambs that have been fattened, at one year and in the best fattening position, weigh up to 100 kg (Karimzadeh *et al.* 2016).The

Lori-Bakhtiari (LB) sheep is a meat breed. The main habitat of this breed is in Chaharmahal and Bakhtiari province.

Having widespread and muscular thighs which are tangled in rams is part of their ecotype. The average birth weight of lambs of this breed is 2.4 to 4.6 kg, depending on gender, the average wool weight of ram and ewe is 2.452 and 1.810 kg, respectively. The fertility rate of this breed is 92% and the rate of twinning is 19.5% (Karimzadeh *et al.* 2016). Growth in animals is controlled by complex systems, in which pituitary hormones play a key role. Among these hormones, growth hormone (GH) and insulin like growth factors (IGFs) along with its binding proteins play key roles in the development and growth of bone and muscle (Adam *et al.* 2004). The IGFs system is a versatile system which includes IGF-I, IGF-II, insulin and its receptor, and six binding proteins (IGFBPs) (Holly, 2004). In humans, mice, cattle and sheep six types of IGFBPs have been identified with prominent influences in the regulation of biological activity of IGFs (Yu *et al.* 1989; Monget *et al.* 1993; Ostecka and Lahovec, 2002). The IGFBPs with different affinity for binding to IGFs named IGFBP1 to IGFBP6 (Hu *et al*. 1999). The IGF-I binding to IGFBPs has several important consequences including the transfer of IGF, protecting it against the destruction, regulate its response to IGF-I receptors and increase its half-life (Hu *et al*. 1999; Jones Clemmons, 1995; Fenwick *et al.* 2008). In most cases, the IGF-I makes a complex with IGFBPs with high affinity (Nagao *et al*. 2001). Because the binding of IGFI to IGFBPs in the blood, its half-life is more than GH. The IGFBPs play most important role in transferring the IGFs to cell surface receptors as well as the effects of IGFs in cell proliferation (Kostecka and Blahovec, 2002). Less than one percent of IGFs in serum are free with short half-life (approximately 10 minutes) (Zapf, 1997). Ten to 25 percent of them existed as dual complex which contains IGFs and one of the IGFBPs, with a low half-life (approximately 30 minutes) (Blum *et al.* 1989). As well as approximately 80 to 85 percent of IGFs in the blood stream to form a triple complex includes IGF-I or IGF- II, IGFBP-3 or IGFBP-5 and its acid labile subunit (ALS) (Baxter *et al.* 1990). In blood stream, the IGFBP3 is the main carrier of IGF protein. The IGFBP2 inhibits the IGF (especially IGF-II), although in certain types of cells can balances the activity of the IGF (Jones Clemmons, 1995). Sharma *et al.* (2013) investigated the polymorphisms of *GHR*, *IGF-I* and *IGFBP3* genes and its association with growth traits in Hindi goats. The results showed that a polymorphism in the *IGF-I*, two in *GHR* and one in *IGFBP3* has significant association with body weight in goats ($P<0.05$).

Lee *et al.* (2005) investigated the *IGFBP2* gene polymorphism and its association with growth and carcass traits in poultry.

The results showed that *IGFBP2* polymorphisms are significantly associated with body weight at 7, 14, 21, 28 and 35 days of age, weight of heart, liver, gizzard, as well as the depth of carcass and pectoral muscle.

Despite some studies on gene polymorphisms of intronic and exonic regions of *IGFBP2* and *IGFBP3*, ever the promoter of these genes has not been considered in molecular study in sheep. Therefore, the aim of this study was to studying genetic polymorphism in promoter region of *IGFBP2* gene in MS and LB breeds, identifying motifs involved in DNA-protein interaction, as well as statistical analysis of the association between the observed polymorphism with some economic traits including body weights at birth (BW), 3 (BW3), 6 (BW6) and 12 (BW12) months of age, and biometric traits including height of the withers (HW), height of the buttocks (HB), chest round (CR), thigh round (TR) and wool weight (WW).

MATERIALS AND METHODS

Sample collection and extraction of DNA

A total of 320 blood samples (120 MS and 200 LB) were collected and DNA was extracted using salting out method (Miller *et al.* 1988).

Primer designing and polymerase chain reaction (PCR)

The upstream regulatory sequence of the studied gene was provided from the NCBI Gene Bank (http://www.ncbi.nlm.nih.gov/) and Ensemble (http://asia.ensembl.org/index.html) (CM001583.1) and its promoter region was determined using the Promoter 2.0 server (http://www.cbs.dtu.dk/services/Promoter/).

One specific primer pair of F:5′-CCTTGCTATGCCTGTATGTC-3′ and R:5′-TCTCTTGGTAGGGATAGGAC-3′ was designed using Oligo 7 software (http://Oligo.software.informer.com/7.5/) and accuracy of primers was checked by the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The PCR was performed in 20 µL reaction mixture containing 100 ng of template DNA, 10 µL of PCR master mix (YektaTjhaizAzma Co., Tehran, Iran), 10 pM of each primers and 7 mL of distilled water. The polymerase chain reaction (PCR) thermal cycling included the primary denaturation at 65 ˚C for 7 min, followed by 34 cycles of denaturation at 95 ˚C for 30 s, 55 ˚C for 45 s (primer annealing), 72 ˚C for 45 s and final extension at 72 ˚C for 7 min. The PCR products were separated by electrophoresed on 1% agarose gel and stained by ethidium bromide.

Genotyping the samples by SSCP assay

Single strand conformation polymorphism (SSCP) assay was used to detect the polymorphisms in promoter regions of studied genes.

In this technique, mutated sequences are detected based on the amount of movement within the polyacrylamide gel that is altered due to a change in single strand conformation (Orita *et al*. 1989).

Amplified fragments of *IGFBP2* gene were analyzed on 10% polyacrylamide gel electrophoresis. Five µL of PCR product was mixed with same volume of SSCP buffer (800 µL formamide, 190 µL glycerol, 2 µL ethylenediaminetetraacetic acid (EDTA) (0.5 M) and 10 µL bromophenol) and denatured for 6 min at 95 ˚C in thermocycler and immediately transferred to cold plate for 10 min and then were run on gel (run condition: 350 volts at 4 ˚C overnight). The polyacrylamide gels were stained with silver nitrate. Different genotypes were determined by counting of the bands on the gel. The population genetic indices at the considered marker site were calculated using POPGEN32 software (Yeh *et al*. 1997).

Sequencing and bioinformatics analysis of sequences

After genotyping, one sample from each genotype was sent to bi-directional sequencing to Bioneer Company (Bioneer Inc., Daejong, South Korea). Before the sequencing, PCR products were purified using DNA purification kit of Roche Company (Roche Molecular Systems, Inc.Mannheim, Germany).

After obtaining the sequencing result, the sequences homology was performed on the NCBI server by BLAST program (http://www.ncbi.nlm.nih.gov/blast).

The alignment of the obtained sequences and their comparison with reference sequences (CM001583.1) from the Gene Bank were done with the CLUSTALW multiple alignment tool of BioEdit software (version 7.0.9.0) (Hall, 1999). The DNASIS MAX software was used to identify DNA motifs.

Statistical analysis and the marker-trait association study

To investigate the association between observed genotypes of *IGFBP2* promoter region and selected traits in MS, the GLM procedure of the SAS statistical software version 9.1 (SAS, 2004) and the following model was used:

$$Y_{ijk} = \mu + G_i + S_j + e_{ijk}$$

Which:

Y_{ijk}: observation of each traits (BW, BW3, 6, 9 and 12, WW, HW, HR, BL, CR and TR).

μ: average of each trait in the population.

G_i: effect of *IGFBP2* genotypes.

S_j: effect of the sex (two levels) of the animal.

e_{ijk}: residual effect.

RESULTS AND DISCUSSION

Detection of polymorphism in studied gene

The DNA was extracted from blood samples and a fragments of 297 bp from *IGFBP2* promoter site was succesfully amplified using PCR (Figure 1).

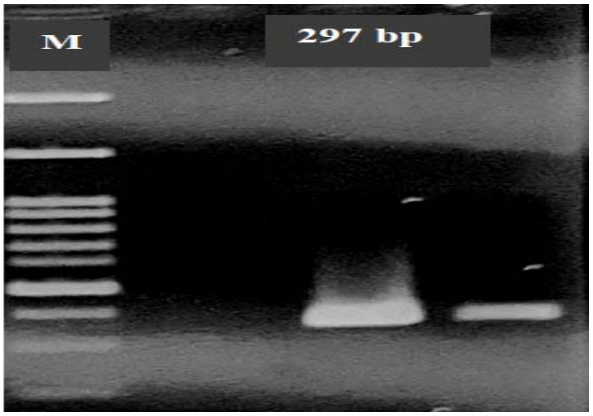

Figure 1 The PCR products of IGFBP2 promoter site in Makuee and Lori-Bakhtiari sheep
M: molecular weight marker (Fermentas, SM0301)

After genotyping by SSCP assay two genotypes AB and BB were observed in both MS and LB (Figure 2). In Table 1 showed the population genetics indices of IGFBP2 promoter site.

The diversity of promoter site of IGFBP2 in LB was more than MS breed according to the effective allele number, the mean heterozygosity and the Shannon index (Shannon and Weaver, 1949). The Shannon index characterizes species diversity in a community.

Sequencing and bioinformatics analysis

As shown in Figure 3 the alignment of the different genotypes of *IGFBP2* promoter site sequences obtained and the reference showed three differences in nucleotide positions 53 (217606533 in reference sequence), 98 (217606579 in reference sequence), and 104 bp (217606595 in reference sequence) (Figure 3).

In addition, analysis of sequences from IGFBP2 promoter site using DNASIS MAX software revealed 10 motifs in studied sites (Tables 2).

Marker-trait association analysis

Investigation of the association between *IGFBP2* promoter genotypes and the studied traits of MS showed that the polymorphism of this site was significantly associated with TR ($P<0.05$), so that BB individuals had a larger TR than AB genotype (Tables 3 and 4).

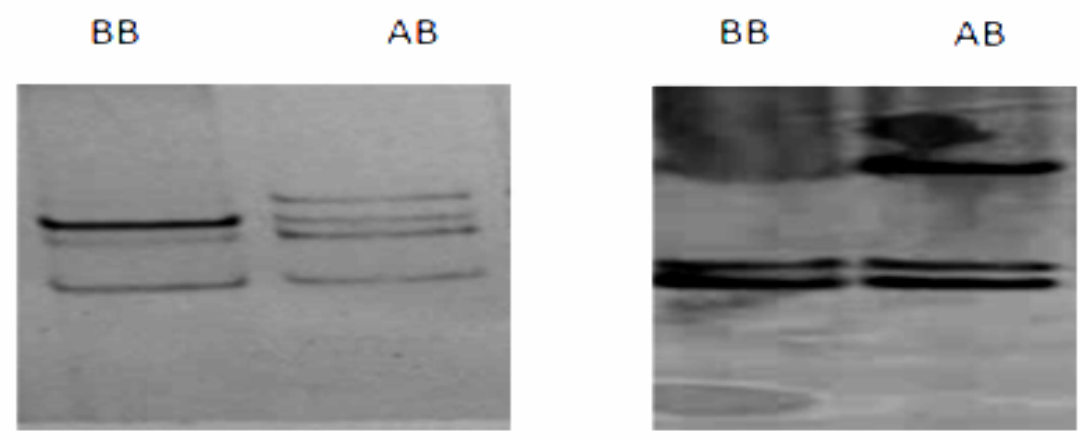

Figure 2 Observed genotype of IGFBP2 promoter site in Makuee (left) and Lori-Bakhtiari (right) sheep

Table 1 Population genetics indices observed for promoter site of IGFBP2 in studied sheep

Shanon index	Mean heterozygosity	N_e	Allelic frequency (%)		Genotype frequency (%)			Breed
			A	B	AA	AB	BB	
0.45	0.28	1.39	17	83	0	34.2	65.8	MS
0.58	0.39	1.65	27	73	0	54	46	LB

N_e: effective number of allele; MS: Makuee sheep and LB: Lori-Bakhtiari sheep.

Figure 3 Comparison of sequence patterns of the observed genotypes in promoter site of the *IGFBP2* gene
REF: sequence of the desired fragment from the Gene Bank (CM001583.1); AB-L and BB-L: genotypes in the Lori-Bakhtiari sheep and AB-M and BB-M genotypes in the Makuee sheep
Y: cytosine or thymine
Black arrows show the specific location of differences between studied sequences

Table 2 DNA motifs identified in different genotypes of IGFBP2 promoter site in Makuee and Lori-Bakhtiari sheep

Motif name	Sequence	Location
bHLH_CS	CANNTG	183-188, 221-226
C_Myb_CS	CMGTTR	122-127
CAP_site	CANYYY	42-47, 59-64, 67-72, 77-82, 93-98, 134-139, 148-153, 268-273
gamma_IRE_CS	CWKKANNY	12-19, 261-268
GMCSF_CS	CATTW	85-89, 93-97
HiNF_A_RS	ATTTNNNNATTT	86-97
HNF_5_CS	TRTTTGY	126-132
NF_IL6_CS	TKNNGNAAK	242-250
TCF_1_CS	MAMAG	106-110
Uteroglobin_HS2.4_CS	RYYWSGTG	219-226

Table 3 P-value results of analysis of variance for association between studied traits and promoter sites of IGFBP2 and sex in Makuee sheep

Loci	Traits										
	BW	BW3	BW6	BW9	BW12	WW	HW	HR	BL	CR	TR
IGFBP2	0.33	0.48	0.54	0.59	0.16	0.70	0.15	0.12	0.69	0.86	**0.018***
Sex	0.36	0.25	0.59	**0.006**	**< 0.0001**	0.59	**0.0038**	**0.0018**	0.40	**0.0059**	**0.04**

* The bold P-values are statistically significant.
BW: body weights at birth; BW 3, 6, 9, and 12: body weights at 3, 6, 9, and 12 months of age; WW: wool weight; HW: height of the withers; HB: height of the buttocks; BL: body length; CR: chest round and TR: thigh round.

In the present study, the sex of studied animals significantly influenced the BW6 and 9, HW, HB, CR, and TR, So that male lambs showed higher values in all of the traits (Tables 3 and 5).

Table 4 Comparison between the means of thigh round trait in association with genotypes of IGFBP2 promoter site in Makuee sheep

IGFBP2	Thigh round (cm)
AB	34.39[b]
BB	35.28[a]
P-value	0.018
SEM	0.20

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In the present study, fragments of 297 bp were successfully amplified from the promoter site of *IGFBP2* gene of MS and LB. The regulatory region especially the promoter of this gene has been considered less in molecular genetic studies in sheep and other farm animals. Results of genotyping of the *IGFBP2* gene promoter site in studied sheep breeds showed polymorphism in both breeds, so that two AB and BB genotypes were observed in both LB and MS breeds with frequencies 54, 46 and 34.2, 65.8%, respectively (Table 1). In both breeds, the highest allele frequency was belonged to allele B with 73 and 83%, respectively. Statistical analysis showed that the polymorphisms of *IGFBP2* gene in MS had a significant effect on the TR trait, so that average of TR trait in individuals with BB genotype was higher than AB genotype.

Although the literature review showed that little research was done on *IGFBP2* gene polymorphism and its association with economic traits in farm animals, the results of this study were in agreement with the results of Lee *et al.* (2005), which revealed that the polymorphisms of the *IGFBP2* gene and its association with growth and carcass traits in poultry. So that the polymorphism of C > T in intron 2 of *IGFBP2* gene was significantly associated with body weight trait at 7 weeks (P<0.05). The gene was also associated with body weight at 7, 14, 21, 28 and 35 days, heart, liver, and gizzard weight, carcass depth and breast muscle mass (Lee *et al.* 2005). So far, IGFBP3 polymorphism and its association with growth-related traits in cattle, sheep and goats have been reported in previous studies (Sharma *et al.* 2013; Kumar *et al.* 2004).

Particularly, Sharma *et al.* (2013) examined the polymorphisms of *IGF-I*, *GHR* and *IGFBP3* genes and their association with growth traits in 309 Indian goats, which showed that polymorphism in *IGFBP3* gene was significantly (P<0.05) associated with body weight of goats in different ages. In addition, Kumar *et al.* (2004) studied the RFLP polymorphism in a 651 bp fragment of IGFBP3 gene in four indigenous sheep breeds using PCR-RFLP technique and reported the fragments of 199, 164, 56, 36, 18, 16 and 8 bp in all four breeds.

In the present study, for a more accurate examination of the promoter region of the *IGFBP2* gene, sequencing of each genotype was done and bioinformatics analysis was performed to identify the nucleotide differences between different genotypes as well as identify the motifs involved in the gene expression regulation process on the sequences. Results of comparing sequences from MS and LB breeds with reference sequence from NCBI Gene Bank indicated the differences between the sequences from studied breeds and reference sequence.

Table 5 Comparison between the means of BW9, BW12, HW, HR, CR and TR in association with sex in Makuee sheep

Sex	BW9 (kg)	BW12 (kg)	HW (cm)	HR (cm)	CR (cm)	TR (cm)
Male	30.43[a]	36.91[a]	64.75[a]	65.97[a]	84.30[a]	35.72[a]
Female	28.02[b]	31.90[b]	62.00[b]	64.09[b]	81.18[b]	34.68[b]
SEM	0.38	0.45	0.45	0.31	0.45	0.20
P-value	0.0006	< 0.0001	0.0038	0.0018	0.0059	0.04

BW 9 and 12: body weights at 9 and 12 months of age; HW: height of the withers; HR: height of the buttocks and TR: thigh round.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Ten different motifs were identified in the amplified fragment of the IGFBP2 promoter site with the highest frequency associated with the CAP_site motif (Table 2). The CAP_site motif is the site of the CAP transcription factor, which is one of the transcriptional stimulatory factors in several loci and affects sugar metabolism, amino acids, transmission processes, protein folding, and so on. The CAP can stimulate operons sensitive to catabolites in the presence of an allosteric cAMP effector (Busby and Ebright, 2001; Lawson *et al*. 2004). The eukaryotic species promoters contain many regulatory motifs that are essential for controlling the transcription of genes. Much evidence suggests that a change in this motif can critically affect the regulation of gene expression. Often, these motifs are binding sites for some proteins such as transcription factors. Some of them are also involved in important processes at the RNA level, including binding to the ribosome, the mRNA process, and transcriptional termination. Today, computer methods have identified a large number of regulatory motifs in the upstream regions of the genes, so that the number of identified motifs is more than the number of known regulatory proteins. Therefore, the precise role of many of these motifs and their binding proteins is still unknown (D'haeseleer, 2006).

CONCLUSION

In the present study, the polymorphisms of *IGFBP2* gene promoter site was studied in association with growth and biometrics traits in sheep. The result of statistical analysis showed a significant association between the different genotypes of this locus with thigh round in MS. The sex of the sheep also significantly affected most of the studied traits. The results of bioinformatics analysis of sequences for each genotype showed 10 different motifs in the promoter of *IGFBP2* gene. However, further research in larger populations and additional observations of genetic regions along with regulatory sites seems to be necessary.

ACKNOWLEDGEMENT

Authors would like to thank staff of Laboratoty for Molecular Genetics (SANRU) for them help and patience.

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