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Single Nucleotide Polymorphism of Ovine Leptin and Insulin-Like Growth Factor 1 Gene in Kivircik Crossbred Ewes

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ABSTRACT

Kivircik crossbred sheep in the Thrace region are commonly grown for meat production. The objective of the present study was to determine the polymorphism of insulin-like growth factor 1 (*IGF1*) and leptin (*LEP*) genes in Kivircik crossbred ewes. Therefore, IGF1/*BfoI* and Lep/*BcnI* polymorphisms were examined by polymerase chain reaction- restriction fragment length polymorphism method. The single nucleotide polymorphism (SNP) in the regulatory region of the *IGF1* gene was detected by amplification of the 294 bp region using specific primers and cleavage with the *BfoI* enzyme. Allele frequencies of A and B were found with 0.915 and 0.085 respectively. The genotype frequencies of *IGF1* gene were 0.85 (AA), 0.13 (AB) and 0.02 (BB). The SNP in the exon 3 of the *LEP* gene was detected by amplification of the 494 bp region using specific primers and cleavage with the BcnI enzyme. The estimated frequencies of three genotypes including GG, GA and AA at Lep/BcnI polymorphism were 0.90, 0.09 and 0.01 and they were 0.055 and 0.945 for A and G alleles, respectively. *LEP* and *IGF1* gene showed polymorphic patterns in Kivircik crossbred sheep population. There was no deviation from Hardy-Weinberg equilibrium (P>0.05) relative to LEP and IGF1 genotypes.

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SK and SA designed the research. SA, SK conducted the experiment. SA analyzed the data. SA and SK wrote the paper. SK revised the paper.

Key words
Leptin, Insulin Like Growth Factor 1, Single nucleotide polymorphism, SNP.

INTRODUCTION

Genetic variations that affecting the physiological pathways are of great interest because these are related with different production traits in farm animals. The development of molecular genetic techniques have accelerated the identification of variations associated with economically important traits. Moreover, molecular genetic studies can determine the genetic breeding potential by identifying genetic variants in different populations (Kök *et al*., 2017). Kivircik sheep breed constitutes almost fifty percent of the sheep population in Turkey. Compared to other native sheep breeds, Kivircik sheep have superior meat quality (Ekiz *et al*., 2009). Therefore, Kivircik sheep is especially known for the delicious taste of the meat (Özcan, 1970). Insulin-like growth factor 1 (IGF1) is a growth factor that plays important role in physiological and metabolic processes in vertebrates (De la Rosa Reyna *et al*., 2010). IGF1 has significant biological functions including increases the stimulating myogenesis, intake of glucose, prevents apoptosis, attend the activation of cell cycle genes, interrupts in the synthesis of DNA, protein, RNA, and in cell proliferation, enhance the synthesis of lipids and stimulating the production of progesterone in granular cells (Etherton, 2004). *IGF1* gene consists of 5 exons located on chromosome 3 in the ovine genome. Although, the structure of the *IGF1* gene differs between species, the 70 amino acid sequence of the expressed protein is the same in all vertebrates (Upton *et al*., 1998). SNPs in *IGF1* gene have associated with daily live weight gain (Casas-Carrillo *et al*., 1997; De la Rosa Reyna *et al*., 2010), live weight (Zhang *et al*., 2008; Trukhachev *et al*., 2016), birth weight (Curi *et al*., 2005; Zhang *et al*., 2008) and carcass traits (Islam *et al*., 2009).

Previous studies have suggested that the *IGF1* gene is significant marker gene for growth traits. He *et al*. (2012) were reported that the polymorphisms in the 5' regulatory region of *IGF1* gene have significant effect on growth traits. Scata *et al*. (2010) also detected two mutations in the 5' regulatory region of ovine *IGF1* gene (G855C and G857A) and one mutation (C271T) in exon 3. Allele T of C271T and haplotype G-T of G855C and C271CT had a positive effect on maintaining a constant yield level during lactation in dairy sheep. Trukachev *et al*. (2016) were reported that SNPs in 5' regulatory region (5363. C>T), 5'UTR (5188.G>C, 5186.G>A) and the first intron (4088.G>A) associated with live weight. Chelongar *et al*. (2014) were shown that the SNP in intron 1 in *IGF1* gene

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Table I.- Primer's sequences and product's sizes.

Gene region	Primer sequence (5'-3')	Annealing temp. (°C)	Product size (bp)	References
IGF1 5' regulatory region	F: TGAGGGGAGCCAATTACAAAGC R: CCGGGCATGAAGACACACACAT	55	294	He *et al*. (2012)
Lep, Exon 3, 170.G>A	F: TGTTGTCCCCTTCCTCCTG R: CCCACATAGGCTCTCTTCTGC	63	463	Bakhtiar *et al*. (2017)

associated with fat thickness (the tick rump). Nazari *et al*. (2016) were suggested that the SNP in exon 1 in the *IGF1* gene could be used as a marker for birth weight.

LEP gene is involved in the control of several important physiological functions including regulation of hematopoiesis, energy expenditure, angiogenesis, wound healing, lipolysis, fetal growth and immune system function (Reicher *et al*., 2011). Nucleotide sequence variants in *LEP* gene have relation with circulating leptin concentration (Buchanan *et al*., 2007; Jonas *et al*., 2016), growth traits (Barzehkar *et al*., 2009; Hajihosseinlo *et al*., 2012) carcass and meat quality traits (Boucher *et al*., 2006; Barzehkar *et al*., 2009) and reproduction traits (Bakhtiar *et al*., 2017). The *LEP* gene is located on the chromosome 4 in the ovine genome that encodes a 167 amino acid leptin protein (Hashemi *et al*., 2011).

Table II.- Restrictions enzymes, restriction product size and genotyping.

Gene	PCR products size (bp)	Restriction enzymes	Restriction products size (bp)	Genotyping
IGF1	294	*Bfo*I (*Bsp*143II Isoschizomers)	100, 194, 294	AB
			100,194	BB
			294	AA
LEP	463	*Bcn*I	193, 270, 463	GA
			193, 270	GG
			463	AA

The *LEP* gene is commonly used for MAS studies because it is associated with many economically important traits. Polymorphisms identified in exon 3 of the sheep *LEP* gene were related to body weight (Hajihosseinlo *et al*., 2012). The SNP in the coding region of the sheep *LEP* gene was correlated with muscle growth (Boucher *et al*., 2006). Two SNPs in intron 2 of sheep *LEP* gene were related to fat-tail percentage and body and carcass weight (Barzehkar *et al*., 2009). *LEP*/*Bcn*I polymorphism (170 G>A) in exon 3 of the sheep *LEP* gene has significant effect on feed conversion ratio and circulating leptin concentration (Jonas *et al*., 2016). This SNP (170 A>G) also was associated with reproduction traits in sheep (Bakhtiar *et al*., 2017).

The present study was designed to investigate SNPs in ovine *IGF1* (C1511G, A1513G, 5' regulatory region) and *LEP* (170 A>G, exon 3) genes in Kivircik crossbreed ewes.

MATERIALS AND METHODS

Tissue samples

A total of 100 Kivircik crossbred (Kivircik x Merino) ewes tissue samples were collected after slaughtering and stored at -20 °C in a deep freezer as far as molecular genetic studies are performed.

DNA amplification and genotyping

PCR-RFLP method was used to determine for *IGF1* (He *et al*., 2012) and *LEP* (Bakhtiar *et al*., 2017) gene polymorphism. The sequences of the primers and the size of the PCR product are given in Table I. Restriction enzymes, the size of restriction products and genotyping are shown in Table II.

All PCR applications were performed with the Phire Tissue Direct PCR Master Mix (ThermoFisher LSG-F170L) in accordance with the manufacturer's instructions. The PCRs for both SNPs were carried out in volumes of 50 µl using; 25 µl Phire Tissue Direct PCR Master Mix, 0,3-0,5 mm tissue sample, 5 µM each primer, and the rest was ddH2O. The amplification was performed at 98°C for 5 min, followed by 40 cycles at 98°C for 5 sec, annealing for 5 sec, 72°C for 20 sec and a final extension of 72°C for 1 min on T100 Thermal Cycler (Biorad). Annealing temperatures are also shown in Table I.

A fragment of 294 bp in the 5' regulatory region of the *IGF1* gene and a fragment of 463 bp in the exon 3 of the *LEP* gene were amplified using the primers given in Table I. The PCR products were subjected to electrophoresis on 2 % agarose/ethidium bromide gel (Aga003R, Bioshop, Canada) in 1× TBE buffer (TBE-001, New Bioscience). Gels were visualized under UV light and documented in WGD30S Molecular Imager apparatus (Wisd).

For *IGF1*/*Bfo*I genotyping, 10 µl of PCR product were digested with 2 µl (20 U) of Fast Digest *Bfo*I (FD2148, ThermoFisher) restriction enzymes at 37°C

for 5 min. For *LEP*.170.G>A genotyping; 10 µl of PCR product were digested with 2 µl (20 U) of BcnI (ER0061, ThermoFisher) restriction enzymes at 37°C for 3 h. The restriction fragments were subjected to electrophoresis on 2 % agarose/ethidium bromide gel in 1× TBE buffer. Gels were visualized under UV light and documented in WGD30S Molecular Imager apparatus (Fig. 1).

Statistical analysis

In this study, The Chi-square test whether genotype frequencies of LEP/*BcnI* and IGF1/BfoI polymorphism were in Hardy Weinberg equilibrium estimated by PopGene Version 1.32 (Yeh *et al*., 1997).

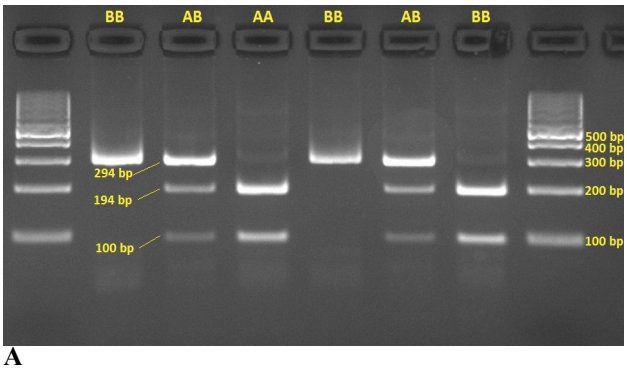
RESULTS AND DISCUSSION

Genotypic distribution and allele frequencies of IGF1/BfoI *polymorphism*

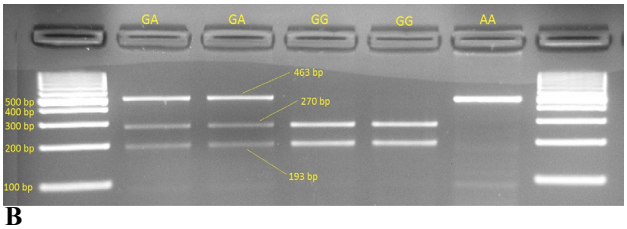
Three genotypes were determined in *IGF1/Bfo*I polymorphism in 5′ regulatory region in Kivircik crossbred ewes (Fig. 1A). The allele frequencies of the *IGF1/Bfo*I polymorphism in 5′ regulatory region were calculated according to Hardy-Weinberg equilibrium (Table III). Allele frequencies of A and B were found with 0.915 and 0.085, respectively. The genotype frequencies of *IGF1* gene were 0.85 (AA), 0.13 (AB) and 0.02 (BB). There was no deviation from Hardy-Weinberg equilibrium (P>0.05) relative to IGF1 genotypes.

5′ regulatory region is one of the polymorphic sites of *IGF1* gene. He *et al*. (2012) determined two polymorphism named as (C1511G and A1513G) in 5′ regulatory region of *IGF1* gene. They reported allele frequencies of A and B in Small Tail Han sheep (0.809-0.191), Hu sheep (0.638-0.362), Texel sheep (0.969-0.031) and Dorset sheep (1.000-0.000), respectively. Trukhachev *et al*. (2016) reported the allele frequencies of 5′ regulatory region of *IGF1* gene as 0.87 (C) and 0.13 (T) in Russian Soviet Merino sheep breed. The allele frequencies for 5′ regulatory region of *IGF1* gene for Small Tail Han sheep, Texel sheep and Russian Soviet Merino sheep breed are similar to our study. The other researchers have reported polymorphisms of *IGF1* gene with different regions. Moradian *et al*. (2013) found the allele frequencies of *IGF1* (Exon 1) as 0.73 (A) and 0.27 (G) in Makoei Sheep.

Niznikowski *et al*. (2014) carried out the study to identify the polymorphisms of *IGF1* (Exon 3) in Polish Lowland Sheep. They reported that there was no polymorphisms of *IGF1* (Exon 3) in Polish Lowland Sheep. Kazemi *et al*. (2011) studied the promoter region of *IGF1* gene in Zel sheep population. The researchers determined the polymorphisms of *IGF1* gene and showed the allele frequencies 0.71 (A) and 0.29 (B). In other study, polymorphisms of *IGF1* (Exon 3) were identified in Pomeranian Coarsewool ewes. The allele frequencies of



A



B

Fig. 2. Restriction products of Ovine *IGF1* gene (A) and Leptin gene (B).

Table III.- Allele and genotype frequences for *IGF1*/*Bfo*I and *LEP*/*Bcn*I polymorphism.

	n	Genotypes			Genotype frequencies			Allel frequencies		$(\chi^2)^1$
		AA	AB	BB	AA	AB	BB	A	B	3.000^{ns}
IGF1										
Observed	100	85	13	2	0.85	0.13	0.12	0.9150	0.0850	
Expected	100	83.6834	15.6332	0.6834	0.83	0.15	0.0068			
LEP										**2.10^{ns}**
Observed	100	90	9	1	0,90	0,09	0.01	0.9450	0.0550	
Expected	100	89.2764	10.4472	0.2764	0.89	0.10	0.0027			

[1] $\chi^2_{0.05;1}$; 3,84 test of Hardy-Weingberg equlibrium; NS, not significant (P > 0.05).

0.205 (C) and 0.795 (T) were given in this study (Proskura and Szewczuk, 2014). Grochowska *et al*. (2017) investigated the polymorphism in 5′ flanking region of the IGF1 gene in in Coloured Polish Merino sheep. The allele frequencies of A and B were found 0.92 and 0.8, respectively.

Genotypic distribution and allele frequencies of LEP/Bcn*I polymorphism*

The genotypes of GG, GA and AA were found in *LEP*/*Bcn*I polymorphism of Exon 3, 170.G>A in Kivircik crossbreed sheep (Fig. IB). The allele frequencies of the *LEP*/*Bcn*I polymorphism were calculated according to Hardy-Weinberg equilibrium (Table III). The estimated frequencies of three genotypes including GG, GA and AA at *LEP*/*Bcn*I polymorphism were 0.90, 0.09 and 0.01 and they were 0.055 and 0.945 for A and G alleles, respectively. There was no deviation from Hardy-Weinberg equilibrium (P>0.05) relative to LEP genotypes. In this study, G allele of *LEP*/*Bcn*I (exon 3) was found homozygous in Kivircik crossbreed sheep population. Similarly, *LEP*/*Bcn*I polymorphism in crossbreed Awassi-Merino sheep were reported by Jonas *et al*. (2016) as 0.08 and 0.92 for A and G, respectively. Mahmoud *et al*. (2014) reported the A and G allele frequencies for Herri sheep breed as 0.086 and 0.914, respectively. In Sanjabi rams, the allele frequencies were reported as 0.76 (G) and 0.24 (A) (Bakhtiar *et al*., 2017). This results are in agreement with the current study.

The *LEP* has different polymorphic sites in sheep breeds. Cauveri *et al*. (2014) determined two polymorphism in the *LEP* Exon 3 (16973 G>A, 17476 C>T) in Nilagiri sheep. The allele frequencies were found as 0.87 (C) and 0.13 (T). The other study carried out in Malpura sheep. *LEP* (Exon 3) T387G locus was found polymorphic. G and T allele frequencies were given 0.82 and 0.18, respectively (Meena *et al*., 2017). Mahmoud *et al*. (2014) have also studied different polymorphic sites of *LEP* gene in Herri sheep breed. There were three non-synonymous polymorphic sites at positions 248 (CTG/CCG-transition), 286 (GTG/TTG-transversion) and 332 (CGG/CAG-transition) and two synonymous polymorphic sites at positions 213 (ACC/GCC transition) and 216 (CCA/CCG transition) determined in this study. The A and G allele frequencies for polymorphic sites at positions 213, 216, 248, 286 and 332 were (0.029-0.971), (0.029-0.971), (0.014-0.986), (0.286-0.714) and (0.114-0.886), respectively.

CONCLUSIONS

The primary aim of this study to identify the polymorphisms of *LEP* and *IGF1* genes in Kivircik crossbreed population. Therefore, the present study provided basic information to understand the genetic diversity of Kivircik crossbred sheep in terms of *IGF1* and *LEP* genes. The genetic improvement of economically important traits can be developed through marker assisted selection. *IGF1* and *LEP* genes are playing pivotal role in growth and metabolism. So, these genes are well known markers for economically important traits of livestock animals. In this study, the *IGF1* and *LEP* genes have showed polymorphic pattern in Kivircik crossbred ewes and provided valuable informations about sheep breeding. Taken together, these informations not only can be used further selection programs in sheep breeding but also contributed to the literature and ongoing studies.

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Statement of conflict of ınterest

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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