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RESEARCH ARTICLE

Determination of BMP-15, BMPR-1B and GDF-9 Gene Mutations of the Indigenous Sheep Breeds in Turkey [1]

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Summary

The high ovulation rate and fertility are very important production traits in sheep production. The aim of the study was to determine the BMP-15, BMPR-1B and GDF-9 gene mutations which are related with the high ovulation with high ovulation rate in Chios, Kivircik, Awassi and Imrose sheep breeds. Fifty sheep from each breed were provided for this study. Blood samples for all the animals were collected in sterile-2 ml tubes containing EDTA. Genomic DNAs were isolated using a standard salt-out method. Thereafter target sites were amplified with polymerase chain reaction (PCR). $FecX^{I}$, $FecX^{H}$, $FecX^{G}$, $FecX^{B}$ mutations in BMP-15 gene, FecB mutation in BMPR-IB gene and $FecG^{H}$ mutation in GDF-9 gene were determined by restriction fragment length polymorphism (RFLP) method. All individuals were non-carriers for these mutations, except $FecX^{G}$ mutation in BMP-15 gene. The animals analyzed in this study were found to be heterozygous carriers for $FecX^{G}$ mutation. There may be other genes affecting fertility in Anatolian breeds. For the genetically explanation of multiple lambing, especially in Chios sheep, it was concluded that different genes should be investigated in the further studies.

Keywords: *BMP-15, BMPR-1B, GDF-9, Mutation*

Türkiye'deki Yerli Koyun Irklarında BMP-15, BMPR-IB ve GDF-9 Genlerindeki Mutasyonların Belirlenmesi

Özet

Koyun yetiştiriciliğinde çoklu gebelik yetiştiriciler tarafından geliştirilmesi istenilen bir özelliktir. Bu çalışmada Türkiye'deki yerli ırk koyunlar arasında yer alan Sakız, Kıvırcık, İvesi ve İmroz ırkı koyunlarda çoklu gebeliği etkilediği ileri sürülen BMP-15, BMPR-1B ve GDF-9 genlerindeki mutasyonlar araştırılması amacıyla yapılmıştır. Bu amaçla her bir ırktan birbiri ile akraba olmayan 50 adet hayvan kullanılmıştır. Tüm hayvanlarda kan toplanması veDNA izolasyonun ardından, BMP-15, BMPR-IB ve GDF-9 genlerindeki hedef bölgeler polimeraz zincir reaksiyonu (PCR) ile çoğaltılmıştır. BMP-15 geninde yer alan $FecX^{I}$ $FecX^{H}$ $FecX^{G}$ $FecX^{B}$ mutasyonları, BMPR-IB geninde yer alan FecB mutasyonu ve GDF-9 geninde yer alan $FecG^{H}$ mutasyonuları restriksiyon parçacık uzunluğu polimorfizmi (restriction fragment length polymorphism- RFLP) metodu kullanılarak belirlenmiştir. Analizi yapılan tüm koyun ırklarında BMP-15 geninde yer alan $FecX^{I}$ (AA), $FecX^{H}$ (AA), $FecX^{B}$ (BB), FecB (AA), mutasyonlarında genotipler homozigot bulunmuşken sadece $FecX^{G}$ (AB) ve GDF-9 geninde yer alan $FecG^{H}$ (AB) mutasyonlarındaki genotipler heterozigot bulunmuştur. Yapılan çalışmalarda heterozigot genotiple çoklu doğum arasında pozitif bir korelasyon olduğu ileri sürülmektedir. Ancak Sakız ırkı koyunların bir batıdaki ortalama doğum sayısı 2 ve üzeri olmasına karşın çalışmanın sonucunda bunun incelenen genlerdeki mutasyonlardan kaynaklanmadığı ileri sürülebilir. Daha sonra yapılacak çalışmalarda özellikle sakız ırkı koyunlarda çoklu doğumun genetik olarak açıklanması amacıyla farklı genlerin incelenmesinin gerekli olduğu sonucuna varılmıştır.

Anahtar sözcükler: *BMP-15, BMPR-1B, GDF-9, Mutasyon*

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INTRODUCTION

Many mammals such as goats and cattle have an ovulation rate of 1 or sometimes 2 whereas others, including rats, mice, hamsters, cats, dogs and pigs have ovulation rates between 4 and 15. Sheep's physiological characteristics including ovulation rate and fecundity can vary greatly due to considerable variation among more than 900 different breeds [1]. It has been known that different ovulation rates in sheep are originated from the effect of a gene group [2]. Piper et al.[3] presented that the Booroola gene (FecB, Bone Morphogenetic Protein Receptor-1B (BMPR-1B), also known as Activin-like Kinase (ALK6), was the first major gene that increases the ovulation rate and litter size in the Booroola strain of Merino sheep [3]. Since the first discovery of FecB gene, several genes affecting ovulation rate and litter size have been detected in sheep. Some of the most important of these genes are Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15, also known as Growth Differentiation Factor 9B (GDF9B) genes [4].

The Booroola gene (FecB) was the first major gene for prolificacy identified in sheep and it is located on chromosome 6 [5,6]. One copy of FecB increases ovulation rate by about 1.5 and two copies (homozygous carriers) increase by about 3 [2]. According to increased ovulation rates, these extra ovulations increase litter sizes about 1 and 1.5 respectively. It has been shown that, effect of FecB is caused by a point mutation in position 830 leading to an arginine/glutamine transition (Q249R) in the bone morphogenetic protein 1B receptor (BMPR-1B) expressed in oocytes and granulose cells [7,8].

It has been known that BMP-15 is essential for female fertility and folliculogenesis in sheep. BMP-15 is a member of the transforming growth factor beta (TGFβ) superfamily which is specifically expressed in oocyte of the developing follicule [4,9]. BMP-15 regulates granulose cell proliferation and differentiation by suppressing follicle-stimulating hormone receptor expression and promoting granulose cell mitosis and all these actions play significant roles in mammalian fertility [10]. Previously, four various mutations were found (Inverdale, Hanna, Belclare and Galway) in the BMP-15 gene and they are located on the sheep X chromosome [9]. Inverdale allele ($FecX^I$) corresponds to a T/A transition at position 896 in the cDNA coding for the BMP-15 [11]. One copy of $FecX^I$ allele increases ovulation rate by about 1.0 and litter size by about 0.6 in heterozygous ewes [12,13]. However, homozygous females have small non-functional ovaries and are infertile [14]. The second mutation in the BMP 15 is $FecX^H$ mutation. Ovulation rate and litter sizes in heterozygous carriers and infertility of homozygous females are identical in Inverdale and Hanna families [15]. Hanna allele ($FecX^H$) corresponds to a C/T transition at position 871 [11] resulting in a premature stop codon at amino acid 23 of the mature protein [8] and this stop codon leads to a loss of bioactivity of the BMP-15 protein [11]. The other two mutations in BMP-15 are $FecX^G$ (Galway) and $FecX^B$ (Belclare) mutations. Galway allele corresponds to a C/T transition at nucleotide 718. $FecX^G$ mutation leads up to a premature stop codon at amino acid 239 of unprocessed protein thus no mature protein is produced [8,11]. Belclare allele corresponds to a G/T transition at nucleotide 1100. Serine to isoleucine change occurs at amino acid 99 of the mature protein (S99I) in $FecX^B$ mutation. GDF9 ($FecG^H$) has been mapped on sheep chromosome 5 [16]. The gene consists of 2 exons seperated by 1126 bp intron and encodes a propeptide containing 453 amino acid residues. The active mature peptide is 135 amino acids long [17]. The GDF9 mutation is a C/T transition at position 1184 of the cDNA substituting a serine for a phenylalanine at position 77 of the mature protein (S77F) [4]. Ovulation rates of heterozygous animals are 2 times greater than those of the wild-type animals. Homozygous animals for this mutation are anovulatory and sterile. However, ovarian follicles can be developed to an abnormal type 5 early antral stage, differing from the homozygous FecX carriers [8].

This study is designed to define the mutations of BMB-1B, BMP-15, GDF-9 genes which are suggested to be related to high ovulation rate in native sheep breed especially in Chios than, Kivircik, Awassi and Imrose sheep; thereby discovering the genetic features will make basis for studies which will be intended for the development of fecundity.

MATERIAL and METHODS

Animals and DNA Extraction

In this study, a total 200 healthy, randomly chosen sheep of the indigenous Turkish sheep breeds were investigated; Kivircik sheep from four flocks (n=50), Chios sheep from three flocks (n=50), Imrose sheep from three flocks (n=50) and Awassi sheep from four flocks (n=50). Information from the breeder was considered in order to avoid family connections. Blood samples were taken into 2 ml sterilized tubes with EDTA from *V. jugularis*. Genomic DNA was isolated by standard salt-out method [18].

PCR-RFLP Analysis

The PCR for *BMPR-1B, BMP15* and *GDF9* were carried out in a final volume of 25 µl containing 1 U Taq DNA polymerase (Fermantas Life Sciences, Canada), 2-2.5 µl 10XPCR buffer (750 mM Tris-HCl (pH 8.0), 200 mM $(NH_4)_2SO_4$,0.1% Tween 20), 1.5 mM $MgCl_2$, 50-100 ng genomic DNA, 100 µM dNTP (Takara, Biotechnology Co, Ltd, Japan) and 10 pmol of each primer.

After digestion with restriction enzymes, all products were separated by using electrophoresis in 2% agarose gel and visualized with ethidium bromide.

BMPR-1B (FecB) Genotyping

The Primer sequences used for the *FecB Ava*II site: *BMPR-1B* F CCA GAG GAC AAT AGC AAA GCA AA and *BMPR-1B* R: CAA GAT GTT TTC ATG CCT CAT CAA CAC GGT C. Amplification program were 94°C for 5 min; 35 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR products were digested with *Ava*II restriction enzyme (Fermantas Life Sciences, Canada) at 37°C for 16 h. After digestion, *FecB* mutation yield 160 and 30 bp fragments (BB) or non-carrier products remain uncut at 190 bp (AA). Heterozygotes should produce fragments of 190, 160 and 30 bp (AB).

BMP-15 Genotyping

The primer sequences, restriction enzymes and product lengths are described in *Table 1*.

The non-carriers for $FecX^{I}$, $FecX^{H}$ and $FecX^{G}$ mutations produce uncut products, whereas non-carriers for $FecX^{B}$ mutation reveal fragments of 122 and 31 bp.

GDF9 ($FecG^{H}$) Genotyping

The Primer sequence for the $FecG^{H}$ *Dde*I site: F: ATG GAT GAT GTT CTG CAC CAT GGT GTG AAC CTG A and R: CTT TAG TCA GCT GAA GTG GGA CAA C. Amplification program were 94°C for 5 min; 35 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR product was digested with *Ava*II restriction enzyme (Fermantas Life Sciences, Canada) at 37°C for 16 h. Digestion of the 139 bp fragment in GDF-9 gene with *Dde*I restriction enzyme can reveal three genotypes. Homozygous carriers should produce an un-cut fragment of 139 bp (BB), the homozygous non-carriers should produce fragments of 108 and 31 bp (AA) and heterozygotes should produce fragments of 139, 108 and 31 bp (AB).

Statistical Analyses

The genotype and allele frequencies of BMB-1B, BMP-15 and GDF-9 gene polymorphisms and G statistic test were used to determine whether the populations are in Hardy-Weinberg equilibrium using POPGENE32 software [19].

RESULTS

The distribution of allele and genotype frequencies of BMB-1B, BMP-15 and GDF-9 genes for Kivircik, Chios, Imrose and Awassi breeds are given in *Table 2*.

In this study, four point mutations in the BMP-15 gene have been investigated in indigenous Anatolian sheep breeds and animals for the $FecX^{I}$, $FecX^{H}$ and $FecX^{B}$

Table 1. *The primer sequences, restriction enzymes and product lengths of $FecX^{I}$, $FecX^{H}$, $FecX^{G}$ and $FecX^{B}$ mutations*
Tablo 1. *$FecX^{I}$, $FecX^{H}$, $FecX^{G}$ ve $FecX^{B}$ mutasyonlarına ait Primer sekansları, restriksiyon enzimleri ve ürün uzunlukları*

Mutation	Primers	Enzyme	Products
$FecX^{I}$	F: GAAGTAACCAGTGTTCCCTCCACCCTTTTCT	*Xba*I	124 and30 bp (BB)
	R: CATGATTGGGAGAATTGAGACC		154, 124 and 30 bp (AB)
			154 bp(AA) non-carrier
$FecX^{H}$	F: TATTTCAATGACACTCAGAG	*Spe*I	218 and 22 bp (BB)
	R: GAGCAATGATCCAAGTGATCCCA		240, 218 and 22 bp (AB)
			240 bp (AA) non-carrier
$FecX^{G}$	F:CACTGTCTTCTTCTTCTTACTGTATTTCAATGAGAC	*Hinf*I	112 and 29 bp (BB)
	R.GATGCAATACTGCCTGCTTG		141,112 ve 29 bp (AB)
			141 bp (AA) non-carrier
$FecX^{B}$	F: GCCTTCCTGTGTCCCTTATAAGTATGTTCCCCTTA	*Dde*I	122 and 31 bp(BB) non-carrier
	R: TTCTTGGGAAACCTGAGCTAGC		153,122 and 31 bp (AB)
			153 bp (AA)

Table 2. *Genotype frequencies of polymorphisms in BMP-15, BMPR-1B and GDF-9 genes in Anatolian sheep breeds*
Tablo2. *Anadolu koyun ırklarında bulunan BMP-15, BMPR-1B ve GDF-9 gen polimorfizmlerinin genotip frekansları*

Breed	n	Genotype Frequency																	
		$FecX^{I}$(BMP-15)			$FecX^{H}$(BMP-15)			$FecX^{B}$(BMP-15)			$FecX^{G}$(BMP-15)			FecB (BMPR-1B)			$FecG^{H}$ (GDF-9)		
		AA	AB	BB	AA	AB	BB	AA	AB	BB	AA	AB	BB	AA	AB	BB	AA	AB	BB
Awassi	50	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0
Chios	50	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0
Imrose	50	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0
Kivircik	50	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0

mutations were homozygous non-careers. Animals were found as heterozygous carrier for only FecX^{G} mutation in the BMP-15 gene. Animals for the FecG^{H} mutation in GDF-9 gene were determined to be heterozygous and non-career animals for FecB mutation in BMPR-1B gene was found in the study.

DISCUSSION

The FecX^{G} mutation in the BMP-15 gene was only observed in some of the Belclare, Cambridge, Garole, Kendapada and Small tailed Han sheeps in previous studies. This mutation leads to a premature stop codon occurs at residue 239 of proprotein. Animals homozygous for any of these four mutations are steril with extremely similar looking ovarian phenotypes because a block in folliculogenesis prevents follicles progressing past the primary stage [4,9]. Ovulation rates are significantly higher in heterozygote animals due to the accelerated follicular development [4]. Hanrahan et al.[4] reported the estimate for the effect of FecX^{G} 0.77 and 1.18 in Belclare ewes and Cambridge ewes, respectively.

For the FecG^{H} mutation in GDF-9 gene all individuals from four breeds have been found to be heterozygous. These results reflected that none of the animals in this study carried the FecG^{H} mutation, because in the presence of the mutation DdeI enzyme could not recognize the restriction site and the fragment remains uncut. The FecG^{H} mutation in the GDF-9 gene was only observed in Belclare and Cambridge breeds. This point mutation resulted in a S77F transition in the mature region of GDF-9 protein. Ewes that have single copy of FecG^{H} mutation, are fertile and they have increased ovulation rates according to the results of Hanrahan et al.[4]. Ghaffari et al.[20] reported that 239 individuals from Shal breed were observed to be non-careers. Generally 20-35% twinning was detected in Shal flocks. They assumed that other genes identified in this breed may affect the fecundity.

In this study, it was observed that only non-career animals for FecB mutation in BMPR-1B gene and our findings for FecB mutation were similar with the results of Karslı and Balcıoğlu [21]. One copy of FecB increases ovulation rate by about 1.5 and two copies (homozygous carriers) increase by about 3. Considering increasement of ovulation rates, these extra ovulations increase litter sizes about 1 and 1.5 respectively [8]. In an earlier study conducted on prolificacy of Awassi sheep in Israel, prolificacy values of non-carrier homozygote, heterozygote and carrier homozygote ewes was found to be 1.28, 1.90 and 1.92, respectively [22]. The Anatolian breeds for this study had a disadvantage in terms of this mutation.

The litter sizes of Kivircik, Imrose, Awassi and Chios breeds were found to be 1.10-1.30, 1.20-1.38, 1.30-1.40 and 1.70-2.30 respectively [23]. In this study, it was investigated six mutations in four Anatolian breeds. All of the individuals in the study were non-careers, except for FecX^{G} mutation. These results showed that Kivircik, Imrose, Awassi and Chios breeds had an advantage for fertility due to heterozygosity for FecX^{G} mutation. However, for other mutations there was no consistence between fertility rates and the frequencies of the mutated alleles.

It was concluded that there may be different mutations significantly affecting fertility rates in Anatolian breeds. The genetic factors affecting fecundity should be investigated further by linkage analyses especially in Chios breed due to its higher twinning rates.

REFERENCES

1. Terrill CE: The distribution of breeds as related to domestication and development of modern genotypes. **In,** Foote WC, Bunch DT (Eds): The Domestication of Sheep: Their Ancestors, Geography, Time Period and People Involved. *Proceedings of A Workshop By The International Sheep and Goat Institude*, 41-112, Utah State University, Logan, 1979

2. Davis GH: Major genes affecting ovulation rate in sheep. *Genet Sel Evol*, 37, S11-S23, 2005.

3. Piper, LR, Bindon BM, Davis GH: The single gene inheritance of the high litter size of the Booroola Merino. **In,** Land RB, Robinson DW (Eds): Genetics of Reproduction in Sheep. pp. 115-125, Butterworths, London, 1985.

4. Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM: Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep *(Ovis aries)*. *Biol Reprod*, 70, 900-909, 2004.

5. Montgomery GW, Lord EA, Penty JM, Dodds KG, Broad TE, Cambridge L, Sunden SL, Stone RT, Crawford AM: The Booroola fecundity (FecB) gene maps to sheep chromosome 6. *Genomics*, 22, 148-153, 1994.

6. Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N, Elsen JM: Mutation in the bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola ewes. *Proc Natl Acad Sci*, 98, 5104-5109, 2001.

7. Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP, Montgomery GW: Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. *Biol Reprod*, 64, 1225-1235, 2001.

8. McNatty KP, Smith P, Moore LG, Reader K, Lun S, Hanrahan JP, Groome NP, Laitinen M, Ritvos O, Juengel JL: Oocyte-expressed genes affecting ovulation rate. *Mol Cell Endocrinol*, 234, 57-66, 2005.

9. Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O: Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet*, 25, 279-283, 2000.

10. Chu MX, Liu ZH, Jiao CL, He YQ, Fang L, Ye SC, Chen GH, Yang JY: Mutations in BMPR-IB and BMP-15 genes are associated with litter size in small tailed Han sheep *(Ovis areas)*. *J Anim Sci*, 85, 598-603, 2007.

11. Fabre S, Pierre A, Mulsant P, Bodin L, DiPasquale E, Persani L, Monget P, Monniaux D: Regulation of ovulation rate in mammals: Contribution of sheep genetic models. *Reprod Biol Endocrinol*, 4, 20, 2006.

12. Davis GH, McEwan JC, Fennessy PF, Dodds KG, Farquhar PA: Evidence for the presence of a major gene influencing ovulation rate on

the X chromosome of sheep. *Biol Reprod*, 44, 620-624, 1991

13. Davis GH, McEwan JC, Fennessy PF, Dodds KG: Discovery of the Inverdale gene (FecX). *Proc New Zealand Soc Anim Prod,* 55, 289-290, 1995.

14. Davis GH, McEwan JC, Fennessy PF, Dodds KG, McNatty KP, Wai-Sum O: Infertility due to bilateral ovarian hypoplasia in sheep homozygous (FecXI FecXI) for the Inverdale prolificacy gene located on the X chromosome. *Biol Reprod*, 46, 636-640, 1992.

15. Davis GH, Bruce GD, Dodds KG: Ovulation rate and litter size of prolific Inverdale (FecXI) and Hanna (FecXH) sheep. *Proc Assoc Adv Anim Breed Genet*, 14, 175-178, 2001.

16. Sadighi M, Bodensteiner KJ, Beattie AE, Galloway SM: Genetic mapping of ovine growth differentiation factor 9 (GDF9) to sheep chromosome 5. *Anim Genet*, 33 (3): 244-245, *2002*.

17. Bodensteiner KJ, **Clay CM**, **Moeller CL, Sawyer HR:** Molecular cloning of the ovine growth/ differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biol Reprod*, **60**, 381-386, 1999.

18. Miller LM, Paszek AA, Schook LB, Louis CF: Denser marker coverage provides greater evidence for carcass trait QTL on SSCP. *Anim Genet,* 29, 60-74, 1998.

19. Yeh F, Yang RC, Boyle T: Popgene (v. 1.32), Microsoft Windows-based freeware for Population Genetic Analysis. Retrieved from http://www.ualberta.ca/~fyeh/Pop32.exe. 2000, *Accessed: 05.11.2009*.

20. Ghaffari M, Nejati-Javaremi A, Rahimi-Mianji G: Lack of polymorphism in the oocyte derived growth factor (GDF9) gene in the Shal breed of sheep. *S Afr J Anim Sci,* 39 (4): 355-360, 2009.

21. Karslı T, Balcıoğlu MS: Türkiye'de yetiştirilen altı yerli koyun ırkında BMPR-IB (Booroola) geninde FecB allel varlığının PCR-RFLP yöntemiyle araştırılması. *Kafkas Univ Vet Fak Derg,*16, 1033-1036, 2010.

22. Gootwine E, Reicher S, Rozov A: Prolificacy and lamb survival at birth in Awassi and Assaf sheep carrying the FecB (Booroola) mutation. *Anim Reprod Sci*, 108, 402-411, 2008.

23. Kaymakçı M, Sönmez R: İleri Koyun Yetiştiriciliği. s. 35, Meta Basım Matbaacılık Hizmetleri, İzmir, 2006.