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Research Article

The Effects of Exon 2 of Inhibin βB Gene and Exon 3 of FSHB Gene on Litter Size in Akkaraman and Bafra Sheep Breeds [1,2]

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Abstract

Inhibin and follicle-stimulating hormone (FSH) are hormones which directly affect ovulation rate in mammals. The aim of this study was to evaluate beta B subunit of inhibin (INHBB: inhibin βB) and beta subunit of follicle-stimulating hormone (FSHB) in terms of prolificacy. For this reason, some polymorphisms of INHBB and FSHB genes were determined with DNA sequencing method in two Turkish native sheep breeds, one of which was Bafra ewes with high prolificacy and the other of which was Akkaraman ewes with low prolificacy. According to GeneBank references, four SNPs, three of which were in INHBB gene and one of which was in FSHB gene, were determined. In Akkaraman ewes, three SNPs, two of which were in INHBB gene and one of which was in FSHB gene, were polymorphic while one SNP in INHBB gene was monomorphic. In Bafra ewes, only one SNP in INHBB gene was polymorphic while the others were monomorphic. All SNPs were investigated in terms of the relationship of litter size within breed and between breeds. In the evaluation of relationship of litter size between breeds, Akkaraman ewes were taken as a low prolific breed to compare Bafra and so it was found that there was no SNP unique in Bafra ewes. In the evaluation of relationship of litter size within breed, variance analyses and logistic regression analyses were used, and each polymorphic SNP was handled individually and together with others (SNPs combination). The effect of SNP and SNPs combination on litter size was insignificant in terms of statistics (P>0.05). Consequently the polymorphisms determined in INHBB and FSHB genes were not related with litter size in Bafra and Akkaraman ewes.

Keywords: *FSHB, Gene, INHBB, Litter size, Sheep*

Akkaraman ve Bafra Koyun Irklarında İnhibin βB Geni Ekzon 2 Bölgesi ve FSHB geni Ekzon 3 Bölgesinin Yavru Verimine Etkisi

Özet

İnhibin ve folikül stimülan hormon (FSH) memelilerde ovulasyon oranını doğrudan etkileyen hormonlardandır. Bu çalışmanın amacı inhibin beta B (INHBB: inhibin βB) ve folikül stimülan hormon B (FSHB) genlerini yavru verimi yönünden değerlendirmektir. Bunun için, Türkiye yerli koyun ırklarından yavru verimi yüksek Bafra koyunları ve yavru verimi düşük Akkaraman koyunlarında INHBB ve FSHB genlerindeki bazı polimorfizmler dizi analiz yöntemi ile tespit edilmiştir. Gen bankası referans dizisine göre üç tanesi INHBB geni içinde bir tanesi FSHB geni içinde olmak üzere toplam dört adet SNP belirlenmiştir. Akkaramanlarda ikisi INHBB geninde biri FSHB geninde olmak üzere üç SNP'de polimorfizm gözlenirken biri monomorfik olarak gözlenmiştir. Bafralarda ise sadece INHBB geninde bir SNP polimorfik olarak gözlenirken diğerleri monomorfik olarak gözlenmiştir. Bu SNP'ler ile yavru verimi arasındaki ilişkiler ırklar arası ve ırk içi araştırılmıştır. Irklar arası değerlendirmede, Akkaraman ırkı yavru verimi düşük ırk olarak alınmış buna göre Bafra ırkına özgü herhangi bir SNP'e rastlanmamıştır. Irk içi değerlendirmede ise polimorfik SNP'lerin tek başlarına ve beraber bulunma durumlarının (SNP kombinasyonları) yavru verimi ile ilişkisine varyans analizi ve lojistik regresyon analizi ile bakılmıştır. Buna göre SNP ve SNP kombinasyonlarının yavru verimi üzerine etkisi istatistiki açıdan önemsiz bulunmuştur (P>0.05). Sonuç olarak INHBB ve FSHB genlerinde tespit edilen polimorfizmlerin Bafra ve Akkaraman koyunlarında yavru verimi ile ilişkili olmadığı belirlenmiştir.

Anahtar sözcükler: *FSHB, Gen, INHBB, Koyun, Yavru verimi*

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INTRODUCTION

Follicle-stimulating hormone (FSH) secreted by hypophysis is the primary hormone regulating mammalian gonad functions [1,2]. FSH included in the follicle growth from early antral stage to ovulation is necessary for the follicle proliferation and survival [3].

FSH has a common α subunit and a possible β subunit. β subunit brings a functional specificity to the hormone [2,4-6]. β subunit of FSH called FSHB has a major effect on FSH level. Therefore, it directly relates with oocyte quantity ovulated [7]. In a study, some polymorphisms on FSHB gene were related with litter size of goats and it was indicated that FSHB could be a candidate gene affecting prolificacy [3].

Inhibin belonging to TGF- β upper family is a growth factor, and a heterodimeric protein which contains a common α subunit and a possible β subunit. The heterodimer of inhibin A consists of α and βA subunits (INHBA), while the heterodimer of inhibin B consists of α and βB subunits (INHBB) [2,8,9].

Oocyte growth depends on FSH level during the oestrus cycle [10]. Inhibin has an important role on the negative feedback mechanism of FSH. When FSH level increases in blood, inhibin is secreted by granulosa cells of antral follicles as a response to decreasing FSH level [11]. Furthermore, FSHB is affected negatively by the presence of inhibin [12]. This mechanism affects the number of oocyte which would ovulate [11]. It was informed that some polymorphisms of INHBB in some sheep breeds were related with multiple births and it was declared that INHBB gene might be a candidate gene effecting on litter size [13].

Bafra sheep breed derived from two Turkish native sheep breeds, Sakiz and Karayaka, has high prolificacy coming from Sakiz sheep breed [14,15]. It was indicated that the litter size of Bafra ewes was between 1.78 and 2.20 [14,15] while the litter size of Akkaraman ewes was between 1.20 and 1.30 [16].

The aims of this study were to detect the polymorphisms in exon 2 and some part of intron closed to exon 2 of INHBB gene, and exon 3 and some part of intron closed to exon 3 of FSHB gene in Akkaraman ewes with low prolificacy and Bafra ewes with high prolificacy and to investigate its relationship with litter size. This study is important in terms of gaining some information about if INHBB and FSHB could be major genes effecting litter size in sheep.

MATERIAL and METHODS

This study was approved by Animal Experimentation Ethics Committee of International Center for Livestock Research and Training (Approval Numbers: 66 and 106).

Animals

Fifty Akkaraman ewes and 49 Bafra ewes were selected for this study. The ages of ewes were 3 to 5 for both breeds. Approximately 200-300 g additional feeding was given to the animals before mating season. After lambing season (February to May), their litter sizes were recorded. While twins in Akkaraman ewes were rarely appeared (litter size: 1.16), multiple births from 2 to 5 in Bafra ewes were frequently appeared (litter size: 2.10). Blood samples were collected from the animals and were stored at -20°C.

DNA Isolation and PCR Amplification

DNA extractions from blood sample were achieved by phenol-chloroform method described by Sambrook and Russell [17]. UV spectrophotometer was used to determine the quality and quantity of DNA extracted. GeneBank references of INHBB gene (Accession number: FJ167874.1) and FSHB gene (Accession number: NC019472.1) were used to design the gene specific primers *(Table 1)*.

For PCR mix, 0.5 µL primer (10 pmol), 2.5 µL 10XTaq Buffer (with KCl), 0.5 µL dNTP (10 µM), 0.3 µL Taq (5U/µL), 5% DMSO, 2 µL genomic DNA (50 ng/µL) were added, and total volume was completed to 25 µL with ddH_2O. Initial denaturation was at 95°C for 3 min, it was followed by denaturation at 95°C for 30 sec, extension was at 72°C for 1 min, final extension was at 72°C for 5 min. The PCR products were controlled in 2% agarose gel with 100bp ladder *(Fig. 1)*.

Sequence PCR

GF-1 PCR Clean-Up Kit (Vivantis, Malaysia) was used for purification of PCR products. Sequence PCR was achieved with two different mixes that were prepared with either F or R primer for each sample. The reaction mixtures consisted of 2 µL primer (3.6 pmol), 2 µL sequencing buffer,

Table 1. *Primers and PCR conditions*
Tablo 1. *Kullanılan primerler ve PCR koşulları*

Primer	Primer Sequence 5´-3´	GC Content	Amplicon Size	Annealing Temperature (°C)	Mg (mM)
Primer-INHBB-Exon-2_F	AGTGGTATTTCTGGTCAGGACGG	52%	897bp	60	1
Primer-INHBB-Exon-2_R	ACTCCTCCACGATCATATTGGGC	52%			
Primer-FSHB-Exon-3_F	TTCAATCCCTGTCTCATTTTG	38%	584bp	53	3
Primer-FSHB-Exon-3_R	AAGCACCCTCGTGTCTGTAAG	52%			

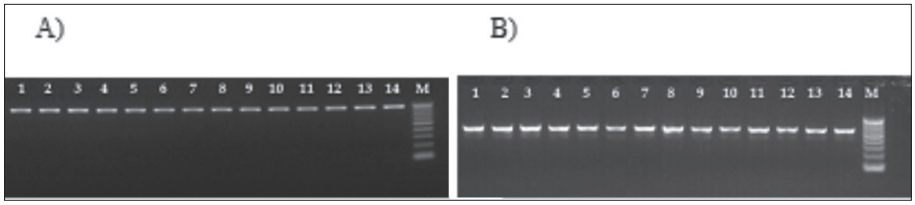
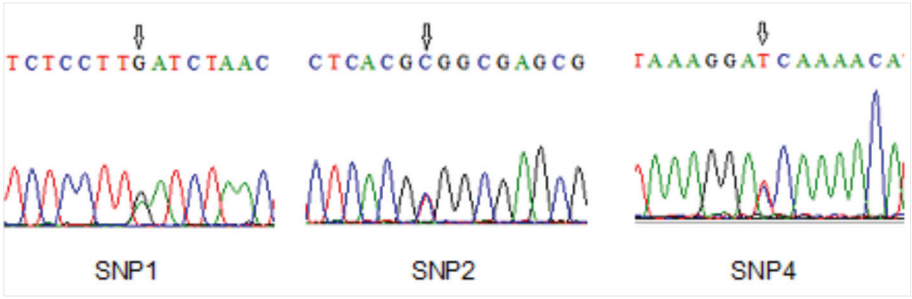


Fig 1. A: INHBB gene, B: FSHB gene (1-14: PCR products, M: 100bp marker)

Şekil 1. A: INHBB geni, B: FSHB geni (1-14: PCR ürünleri, M: 100bp'lik belirteç)

Fig 2. Heterozigosities of SNPs

Şekil 2. Heterozigotluk gözlenen SNP'ler



2 µL sequencing standard, 2 µL PCR product and 2 µL ddH_2O. Initial denaturation was at 96°C for 1 min, the rest of denaturation was at 96°C for 10 sec, annealing was at 50°C for 5 sec, extension was at 60°C for 4 min. The PCR products were precipitated to get rid of salt and heavy metals. Sequence was achieved by the DNA sequencing system.

Bioinformatics and Statistical Analyses

All sequence data were aligned with Bioedit software [18] and the polymorphisms were determined by using GeneBank reference sequences of INHBB gene (Accession number: FJ167874.1) and FSHB gene (Accession number: NC019472.1). Hence a number was given to each polymorphism determined. Then each SNP was controlled if there was the same SNP reported to GeneBank. PLINK 1.9 software [19] was used to compute minor allele frequencies (MAF), expected heterozygosity, observed heterozygosity and Hardy-Weinberg equilibrium.

The relationship of these polymorphisms with litter size was evaluated within breed and between breeds. In the evaluation of relationship of litter size between breeds, Akkaraman ewes were used as low prolific group and all the polymorphisms were controlled if any polymorphisms were specific to Bafra ewes. In the evaluation of relationship of litter size within breed, variance analyses and logistic regression analyses were used to relate the polymorphisms with litter size. Each polymorphism was evaluated individually and together with others (SNPs combination). In the evaluation of polymorphisms individually, genotypes of a polymorphic SNP observed in a breed was analyzed to relate with litter size statistically. In the evaluation of polymorphisms together, a combination was created with a genotype of a polymorphic SNP with the other genotype of the other polymorphic SNPs. SNPs combinations were evaluated with statistical analyses to relate with litter size. Binary model was used for logistic regression analyses.

RESULTS

Four SNPs were determined, three of which were in INHBB gene and one of which was in FSHB gene. SNPs determined were coded as 1, 2, 3, 4. Thus SNP1 was at nucleotide position 1186 of the reference sequence of INHBB and AA, GA, GG genotypes were determined in both breeds. SNP2 was at nucleotide position 1495 in the reference sequence of INHBB and CC, CT, TT genotypes was determined in Akkaraman ewes, while only CC genotype was determined in Bafra ewes. SNP3 was determined at nucleotide position 1611 in the reference sequence of INHBB and only CC genotype which caused the amino acid change from Glutamate (Glu: E) to Alanine (Ala: A) was found in both breeds. SNP4 was determined at nucleotide position 2691 in the reference sequence of FSHB, and CC, CT, TT genotypes were found in Akkaraman ewes while only CC genotype was determined in Bafra ewes. Accordingly, SNP1 was observed in intron of INHBB while SNP2 and SNP3 were observed in exon 2 of INHBB. SNP4 was observed in intron of FSHB. The heterozygosities of SNP1, SNP2 and SNP4 are presented in *Fig. 2*. Minor allele frequency (MAF), expected heterozygosity, observed heterozygosity and Hardy-Weinberg equilibrium of SNPs are presented in *Table 2*.

When SNPs were evaluated between breeds, there was no SNPs particular to Bafra ewes. Thus, the high prolificacy which is the genetic feature in Bafra ewes was not able to be related with the SNPs in question.

Variance analyses and logistic regression analyses were used to evaluate SNPs within breed. In Bafra breed, only SNP1 could be evaluated as polymorphic because the other SNPs were found monomorphic *(Table 3)*. And there was no significant relationship between genotypes of SNP1 and litter size in Bafra ewes (P>0.05). In Akkaraman breed, SNP3 has been found monomorphic, for this reason it was not evaluated. Hence, SNP1, SNP2 and SNP4 were evaluated individually and in SNPs combination *(Table 4)*.

Table 2. *A: Allele evaluation of Akkaraman ewes, B: Allele evaluation of Bafra ewes (CHR: chromosome, A1: minor allele, A2: major allele, NMA: non-missing allel, GENO: genotype ratio, Ho: observed heterozygosity, He: expected heterozygosity, p: p values for Hardy-Weinberg equilibrium)*

Tablo 2. *A: Akkaraman koyunlarının allel değerlendirmesi, B: Bafra koyunlarının allel değerlendirmesi (CHR: kromozom, A1: minör allel, A2: majör allel, NMA: okunan allel sayısı, GENO: genotip oranı, Ho: gözlenen heterozigotluk, He: beklenen heterozigotluk, p: Hardy-Weinberg p değeri)*

A								
CHR	**SNP**	**A1**	**A2**	**MAF**	**NMA**	**GENO**	**Ho**	**He**	**p**
2	SNP1	G	A	0.4082	98	8/24/17	0.4898	0.4831	1
2	SNP2	T	C	0.2500	100	5/15/30	0.3000	0.3750	0.1495
2	SNP3	0	C	0	100	0/0/50	0	0	1
15	SNP4	T	C	0.1700	100	1/15/34	0.3000	0.2822	1
B									
CHR	**SNP**	**A1**	**A2**	**MAF**	**NMA**	**GENO**	**Ho**	**He**	**p**
2	SNP1	G	A	0.3878	98	7/24/18	0.4898	0.4748	1
2	SNP2	0	C	0	98	0/0/49	0	0	1
2	SNP3	0	C	0	98	0/0/49	0	0	1
15	SNP4	0	C	0	98	0/0/49	0	0	1

Table 3. *Evaluation of Bafra ewes within breed*

Tablo 3. *Bafra koyunlarının ırk içi değerlendirilmesi*

SNPs	Genotypes	n	Litter Size Average	Variance Analyses P Value	Logistic Regression P Value
SNP 1	SNP1=GA	24	2.13	0.888	0.720
	SNP1=AA	18	2.17		0.417
	SNP1=GG	7	2.00		0.999

DISCUSSION

In this study, SNPs determined in INHBB gene and in FSHB gene were evaluated. Only few investigators had previously appealed to DNA sequencing method to study INHBB and FSHB genes as candidate genes of prolificacy in small ruminant [13,20].

SNP1 was matched up with the SNP whose accession number was rs409298247 while SNP4 was matched up with the SNP at the position 261 of the nucleotide sequence whose accession number was AY853254 in GeneBank. It was remarkable that SNP2 and SNP3 were the SNPs had not been notified to GeneBank.

All SNPs were in Hardy-Weinberg equilibrium within breeds. SNP1 was a common SNP (MAF>0.05) in both sheep breeds. Therefore SNP1 can be used for other association studies in both breeds. SNP2 and SNP4 were common SNPs (MAF>0.05) in Akkaraman sheep breed. Therefore SNP2 and SNP4 can be used for other association studies in Akkaraman sheep breed.

SNP2 and SNP3 were determined in the exon. It did not change the amino acid structure because SNP2 which was in exon 2 of INHBB gene was silent in codon. SNP3 which was in exon 2 of INHBB was monomorphic in both breeds and caused an amino acid change from Glutamate (Glu: E) to Alanine (Ala: A). However, it was found that this change did not have any effects on litter size. We could not discover any polymorphisms on exon 3 of FSHB gene.

All SNPs even polymorphic or not were evaluated between breeds and it was not found any SNPs which was specific to only Bafra breed. In the evaluation of within breed, polymorphic SNPs and SNPs combinations did not have any effects on the differences of litter size statistically.

Zhang et al.[20] detected a change from Glutamine (Gln) to Arginine (Arg) at exon 3 of FSHB in goat. They called the genotypes of the polymorphism as AA, AB, BB in four goat breeds and declared that this polymorphism had significant effect on litter size. But we could not define any polymorphism at the same nucleotide position in the present study.

Chu et al.[13] defined a SNP at intron of INHBB gene in prolific Hu sheep. The researchers called the genotypes of this SNP as AA, AB, BB and notified that BB genotype had significant effect on litter size compared with AA genotype. But we could not evaluate this polymorphism because this nucleotide position was not between the sequence borders in the present study. SNP1, SNP2 and SNP3 that we determined were not revealed by these researchers.

As a result, it was concluded that the SNPs, which we have evaluated, could not be candidate genes affecting litter size for Akkaraman and Bafra ewes. It could be recommended

Table 4. *Evaluation of Akkaraman ewes within breed. a: One way ANOVA, b: Kruskal Wallis*
Tablo 4. *Akkaraman koyunlarının ırk içi değerlendirilmesi. a: Tek yönlü ANOVA, b: Kruskal Wallis*

SNPs	Genotypes	n	Litter Size Average	Variance Analyses P Value	Logistic Regression P Value
SNP1	GA	25	1.16	0.995[a]	0.564
	AA	17	1.16		0.302
	GG	8	1.17		1.000
SNP2	CT	14	1.17	0.512[a]	0.475
	CC	31	1.14		0.478
	TT	5	1.27		0.230
SNP4	CT	15	1.13	0.388[b]	0.827
	CC	33	1.18		0.999
	TT	2	1.00		0.999
SNP1, SNP2 combination	GA, CT	13	1.18	0.671[b]	0.853
	GA, CC	12	1.14		0.362
	GG, CT	1	1.00		0.362
	GG, CC	2	1.00		1.000
	GG, TT	5	1.27		0.999
	AA, CC	17	1.16		0.174
SNP2, SNP4 combination	CC, CT	12	1.14	0.472[b]	0.700
	CC, CC	18	1.15		0.910
	CC, TT	1	1.00		-
	TT, CT	3	1.11		0.380
	TT, CC	2	1.50		-
	CT, CC	13	1.18		0.713
	CT, CT	1	1.00		-
SNP1, SNP4 combination	GA, CT	4	1.33	0.256[b]	0.722
	GA, CC	19	1.14		0.130
	GA, TT	2	1.00		-
	GG, CT	4	1.08		0.464
	GG, CC	3	1.33		0.482
	GG, TT	1	1.00		-
	AA, CT	7	1.05		0.352
	AA, CC	10	1.23		0.788
SNP1, SNP2, SNP4 combination	GA, CT, CC	13	1.18	0.182[b]	0.954
	GA, CC, CT	4	1.33		0.383
	GA, CC, CC	7	1.05		0.392
	GA, CC, TT	1	1.00		-
	GG, CT, TT	1	1.00		-
	GG, CC, CT	1	1.00		-
	GG, CC, CC	1	1.00		-
	GG, TT, CT	3	1.11		0.999
	GG, TT, CC	2	1.50		-
	AA, CC, CT	7	1.05		0.392
	AA, CC, CC	10	1.23		0.999

that subsequent studies on prolificacy in Bafra and Akkaraman ewes should focus on the other exons and introns of these genes or other possible candidate genes.

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