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Genetic variants of the bone morphogenetic protein gene and its association with estrogen and progesterone levels with litter size in Awassi ewes

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Abstract

This research aimed to assess the genetic polymorphism of the *BMP15* gene concerning sex hormone levels and birth type in Awassi ewes. The genome DNA of 138 Awassi ewes was isolated (52 ewes produced a singleton and 86 produced twins). The *BMP15* gene exon-2 was amplifiable by polymerase chain reaction (PCR), so two genotypes were identified based on 141 bp amplicons: TT and TA. A sequencing reaction revealed a novel mutation, c.50980646T>A, in the TA genotype. This single nucleotide polymorphism (SNP) showed a high association ($P \leq 0.01$) with sex hormone levels and litter size, sheep containing this SNP had higher levels of sex hormones and larger litter sizes than sheep without it. Ewes with the TA genotype had a 1.89 litter size than their TT counterparts. Logistic regression confirmed that the c.50980646T>A SNP increased litter size. In conclusion, the c.50980646T>A SNP appears to be significantly related to reproductive traits (especially sex hormone levels and litter size) in Awassi sheep. With these results, mutations of the *BMP15* gene are suitable for developing marker-assisted selection programs to increase Awassi sheep litter size.

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Introduction

One of the critical objectives of sheep breeding worldwide is to increase prolificacy. Breeding production in sheep is greatly affected because most sheep produce single lambs, and only a small number produce twin lambs (1,2). It is well known that reproductive traits are determined genetically by minor and significant gene mutations that affect ovulation and birth type (3). Several fecundity genes are associated with sheep reproduction, one of them is the bone morphogenetic protein (*BMP*) gene. Nevertheless, mutations in these genes that affect fecundity differ in their influence on reproductive efficiency and birth type (4). Bone morphogenetic protein (*BMP15*) is a reproductive gene that encodes a protein that affects the development of follicles and the reproduction of animals. As a result, geneticists classify this gene as a fecundity gene because of its strong association with ovulation rate and fecundity (5). Sheep have the *BMP15* gene on their X chromosome, including two exons that encode 393 amino acids as a prepropeptide. BMP15 contains 125 amino acids in its mature active form (6). Oocytes express the BMP15 protein, a member of the transforming growth factor family (TGFβ), to regulate granulosa cell (GC) function during ovarian follicle development (7). The protein plays a specific function in the maturation of primary follicles in mono- and polyovulatory species (8). Ovarian granulosa cell proliferation and differentiation are dependent on BMP15 during follicular development. By inhibiting the expression of FSH receptors, it stimulates granulosa cell division (5). The *BMP15* gene has been found to have multiple mutations that affect fecundity in sheep. Ewes with heterozygous mutants can ovulate more frequently, while those with homozygous mutants are sterile due to the deficiency of ovaries' primary

follicles (3). Wang *et al*. (9) reported higher fertility and infertility rates among Belclare and Cambridge sheep to belong to a c.718C>T mutation in the *BMP15* gene. A subsequent study in GC demonstrated that BMP15 inhibits progesterone production induced by FSH without affecting estradiol (10). It has been observed that Fec^{XI} heterozygotes develop more differentiated follicles, decreased follicle granules, and increased sensitivity to LH of the granulosa cells as follicles mature (4). Grivette and Olkuska ewes breed in France and Poland, the non-conservative mutation Fec^{XG} and Fec^{XO} have been identified as hyper-prolific (11). Moreover, the *BMP15* gene mutation causes five naturally occurring mutations reported to modulate ovarian function in Lacaune sheep (12). As a result of the significant effect *BMP15* polymorphism has on ovarian function, litter size, and reproduction in livestock, *BMP15* is regarded as one of the crucial genes that influence reproduction in livestock (13). In the light of the above results, no previous reports had been published on the association of *BMP15* polymorphism with sex hormone levels and litter size in Awassi sheep. Thus, this research aimed to investigate whether variations of the *BMP15* gene could affect sex hormone levels and litter size in Awassi ewes.

Materials and methods

Animal resources

According to the international guidelines for the care and use of animals, the study was approved and carried out at Al-Qasim Green University using Awassi ewes from July 2020 up to March 2021. The study involved 138 sexually mature ewes (52 ewes with a single offspring and 86 ewes with twin offspring), aged between 2 - and three years old. Our collection of animals came from two sheep farms in Iraq, one in Babylon and one in Karbala. Each year, the ewe's pedigree information was updated regarding the identification number of the lambs, their sires, dams, and their date of birth. After parturition, ewes were grouped according to their birth types: those with a single lamb and those with twins. Seasonal grass was provided for the sheep, and concentrated feed was given to them daily (2.5% of their body weight, 59% barely, 40% bran, and 1% salt) and freshwater. Blood samples were collected from the sheep's jugular vein using vacutainer tubes containing EDTA. Centrifugation at 3,000 rpm for 15 minutes at room temperature separated serum from blood; samples were frozen at –20°C for hormone testing. As well as the third, fourth, and fifth months of pregnancy, sexual hormone concentrations were assessed during the postpartum period (from 10-20 days after giving birth). The ELISA kit (with catalog numbers E0047Sh and E0015Sh) was purchased from Bioassay Technology Laboratory Company for measuring estrogen and progesterone hormone.

DNA extraction, genotyping, and sequencing reaction

The study was presented in the Biotechnology Lab, College of Agriculture, Al-Qasim Green University. Genomic DNA was extracted from whole blood using a rapid salting-out method (14). DNA samples were tested using a NanodropμLITE spectrophotometer (Biodrop, UK) to perform a polymerase chain reaction (PCR). Traditional PCR was performed using 10 pmol of each primer, 50 ng of genomic DNA, 50 dNTPs, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, 1 U Top DNA polymerase. An amplicon of 141 bp in exon 2 of the *BMP15* gene was amplified with one specific primer pair (Fig. 1, A). Primer sequence of the B2 amplicon was F-5′-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3′ and B2-R-5′-GATGCAATA CTGCCTGCTTG-3′ (8). This experiment was conducted using the AccuPower PCR PreMix from Bioneer (South Korea). For the PCR reaction, the steps were denaturation at 94°C for 4 min, annealing at 63.1°C for 45 sec, elongation at 72°C for 45 sec, and a final extension at 72°C for 5 min. A 2 % agarose gel was used to visualize PCR products (15).

Polymerase chain reaction-SSCP (PCR-SSCP) was conducted (16). The SSCP denaturing-loading buffer was applied to each PCR amplicon in equal amounts. Using 8% polyacrylamide gels in 0.5X TBE buffer, the denatured samples were loaded after 7 minutes of denaturation followed by 10 minutes of cooling on wet ice. Electrophoresis was performed at room temperature for 4 hours with a constant current and voltage of 200 mA and 100 V. The gel staining was performed according to Byun *et al*. (17). Detected nucleic acid polymorphisms were sequenced in Korea at Macrogen Geumchen and then edited and visualized by Bioedit (version 7.1) (DNASTAR, Madison) and SnapGene Viewer ver. 4.0.4 (http://www.snapgene.com). Furthermore, Ensemble genome browser 96 [available at] verified the novelty of the observed *BMP15* variants.

Analysis of statistical data

ANOVA-repeated measures were used to analyze mean hormone concentrations among genotypes (GLM procedure of SPSS, v 23). The following model was used; $Y_{ijk} = \mu + G_i + P_j + (GP)_{ij} + p_{k(j)} + e_{ijk}$, where μ is the overall mean, Gi is the main effect of genotype (fixed w/$\sum i\ G_i$=0), P_j is the main effect of the physiological stage (gestation and post-parturition) (fixed w/$\sum j\ P_j$ =0), $(GP)_{ij}$ is the interaction effect, $p_{k(j)}$ is the main effect of subjects' $N\ (0, \sigma^2{}_p)$, and e_{ijk} is random error assumed $e_{ijk} \sim N\ (0; \sigma^2)$. Tukey-Kramer tests were used to compare means for significant main factors. The significance of each analysis was determined by P-values lower than 0.05. Genetic data, allele frequencies, and Hardy-Weinberg deviation were measured using Popgen32 software, version 1.31. Associations between *BMP15*

genotypes and litter size were investigated using binary logistic regression analysis.

Ethical approve

The study was approved and carried out at Al-Qasim Green University using Awassi ewes with approval number (Agri, No. 020,7,18) during the period July 2020 to March 2021 according to the international guidelines for the care and use of animals.

Results

Genotyping of *BMP15* gene

All 138 samples were tested using polymerase chain reaction (PCR) 52 ewes with singletons and 86 ewes with twins (Figure 1). PCR-SSCP genotyping identified TT and TA genotypes (Figure 1); sequencing of the SSCP variants confirmed that only one contained the T82A SNP, indicating heterogeneity in exon 2. In light of the substitution c.50980646T,>A SNP or T82A, the SSCP variants with homozygous T/T genotype values were assigned genotype values TT and those with heterozygous T/A genotype values TA (Figure 1). Based on the genetic diversity of the T82A SNP, the genotype TT was more prevalent than the genotype TA (Table 1). A comparative analysis of the genetic diversity of the *BMP15* gene revealed that TT genotypes were more prevalent than others (6.62, n = 86) than TA genotypes (0.38, n = 52). A Chi-square test indicated significant deviance from the HWE at the T82A SNP locus for polymorphisms within the *BMP15* gene (P≤0.05) (Table 1).

Association analysis of *BMP15* genotypes with sex hormones levels of Awassi ewes

Regarding the effects of *BMP15* genotypes on sex hormone levels of Awassi ewes, the results indicated significant differences (*P*≤0.01) in estradiol and progesterone concentrations during pregnancy and post-parturition months between the observed genotypes (Table 2). The TA genotype was associated with significantly higher levels (*P*≤0.01) of estradiol and progesterone than those with the TT genotype. Logistic regression analysis examined the relationship between the T82A genotype and litter size. Sheep of TA genotype had a significantly higher birth type (*P*≤0.01) than those with TT genotype (Table 3).

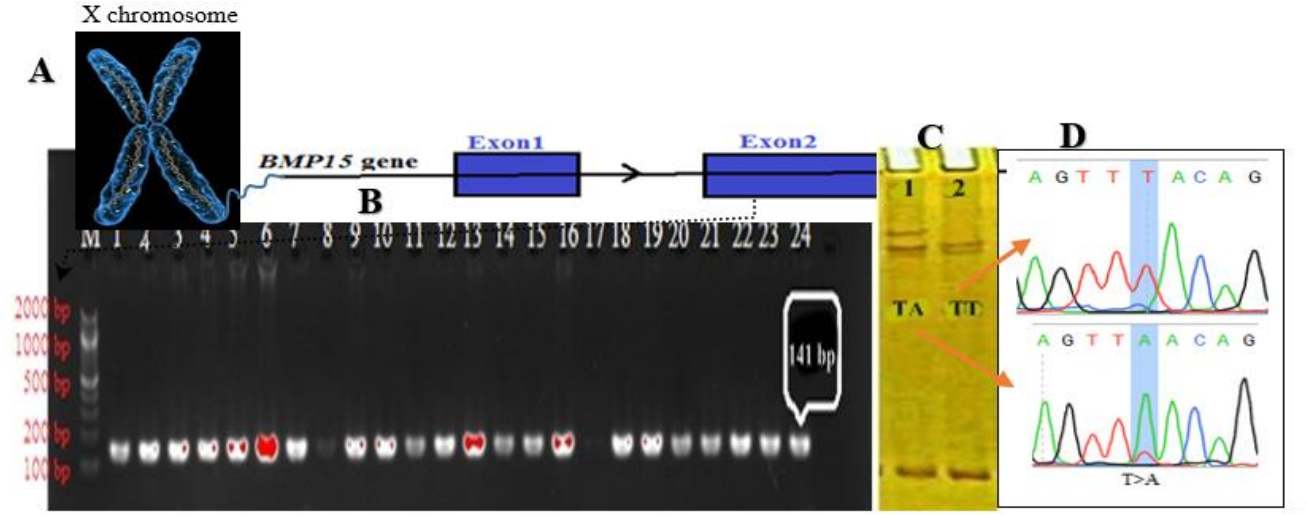

Figure 1: Genotyping of *BMP15* gene. (A) The precise genetic position of the B2 fragment that amplified the *BMP15* exon-2 has been described by GenBank acc. no. NC_019484. B) Agarose gel electrophoresis of PCR products amplified from the *BMP15* gene. C) SSCP genotyping of *BMP15* gene (exon 2) PCR products showing TT and TA genotypes in Awassi ewes. D) Sequencing the polymorphic B2 fragment revealed a novel mutation at T82A SNP.

Table 1: Genetic diversity and genotype parameters for the *BMP15* gene

Genotype frequencies		Allele frequencies		*Ho*	*He*	Ne	PIC	χ^2
TT	TA	T	A	0.38	0.30	1.44	0.36	5.33
0.62	0.38	0.81	0.19					

Abbreviations: *Ho* – observed heterozygosity, *He* – expected heterozygosity, Ne- adequate allele number, PIC– polymorphism information content, χ^2 – chi-square. * All Chi-square tests have one degree of freedom and are within the significance level of P≤0.05.

Table 2: The association between *BMP15* genetic polymorphism with sex hormones levels and litter size in Awassi ewes

Hormones	Months	Genotype (LSM ± SE)		*P*-value
		TT (86)	TA (52)	
Estrogen (pg/ml)	3rd Month	21.01 ± 1.14[b]	54.17 ± 1.27[a]	0.01
	4th Month	24.45 ± 1.11[b]	57.26 ± 1.26[a]	0.01
	5th Month	42.36 ± 1.23[a]	70.21 ± 2.34[b]	0.01
	Post-parturition	13.10 ± 1.60[a]	14.60 ± 1.48[a]	0.31
Progesterone (ng/ml)	3rd Month	3.49 ± 0.14[b]	10.74 ± 1.78[a]	0.01
	4th Month	4.15 ± 0.12[b]	10.03 ± 1.61[a]	0.01
	5th Month	2.34 ± 0.01[b]	5.92 ± 0.31[a]	0.01
	Post-parturition	0.94 ± 0.02[b]	2.68 ± 0.03[a]	0.01

LSM ± SE, least square means ± Standard error. [A,b] Significant differences in means represent differences in the same raw within each classification.

Table 3: Logistic regression analysis of *BMP15* T82A with litter size in Awassi ewes

Genotype	Litter size (LSM±SE)	Logistic Regression analysis		
		β	Odds ratio (95%CI)	*P*-value
TT	1.47 ± 0.04[b]	1.00	Reference	0.001
TA	1.89 ± 0.01[a]	2.78	2.97 (2.32- 6.43)	

LSM ± SE, Least square means ± Standard error, β: Regression coefficients, [a,b] Significant differences in means represent differences in the same column within each classification, the *P*-value with statistical significance are indicated in bold numbers, CI: confidence interval. The litter size is calculated by dividing the total lambs born /lambing ewe.

Discussion

BMP15 is the autocrine and paracrine factor influencing the development of follicles and controlling ovulation rate (5). Defects in the *BMP15* gene have been found to affect folliculogenesis in ewes. Mutations of *BMP15* cause infertility in homozygous ewes, while heterozygous ewes are more fertile and have larger litters (3). Natural *BMP15* point mutations in heterozygous ewes make them more prone to ovulation (4). According to Garcia-Guerra *et al*. (18), heterozygous genotypes of *BMP15* enhance antral follicle development and generate several oocytes at once, which can enhance their sensitivity to FSH and LH. Hence, *BMP15* is critical in determining the number and size of sheep's litter, and it is a potential genetic marker for assessing litter size (5). BMP15 is known to promote the maturation of follicles from the primordial and gonadotropin independent phases, regulate the sensitivity of GCs to FSH action, prevent the cell death of GCs, increase oocyte processing and development, and regulate ovulation quotas (7). Furthermore, the *BMP15* gene underregulates the steroidogenic acute regulatory protein (StAR) synthesis to inhibit follicular luteinization. It induces granular cell differentiation and increases follicular maturation in mutation ewes to increase ovulation. Due to the sudden loss of oocyte factors after ovulation, StAR expression is elevated, leading to progesterone production (19).

In many studies, the *BMP15* gene has proven polymorphic among sheep breeds. Ali *et al*. (20) identified *BMP15* genotyping using *Spe*I and *Hinf*I /PCR-RFLP in Karadi Sheep and revealed Fec^{XH} and Fec^{XG} point Mutations. Chantepie *et al*. (13) determined the variant g.50977717T > A of the *BMP15* gene in Noire du Velay sheep and determined three genotypes TT, TA, and AA. Furthermore, genetic variation of the *BMP15* gene exon 2 in purebred Kermanis and crossbred Romanov × Kermani sheep are identified and assigned eight genotypes in which AA and BB genotypes are the most frequent (21).

The present study indicated a significantly larger litter size ($P \leq 0.01$) in the TA genotypes and higher estradiol and progesterone levels than those with the TT genotype. In livestock, reproductive hormone levels can assess reproductive performance (19). In response to the increased levels of reproductive hormones, the ovarian follicles develop and mature more rapidly, which leads to an increased litter size (5). The high levels of reproductive hormones cause increased litter size due to increased development and maturation of ovarian follicles. Physiologically, estrogen is generated by granulosa cells of preovulatory follicles by aromatizing androgen in the follicles growing (22). Granulosa cells in follicles are stimulated to proliferate by this hormone. In addition, it enhances the action of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (5). This allows follicle growth because an increase in granulosa cell number is

directly responsible for the growth of follicles rather than antrum formation. As well as its role in facilitating granulosa cell differentiation, estrogen induces receptor systems for FSH, LH, and prolactin, influencing post-receptor mechanisms (23).

Moreover, the increase in corpora lutea is associated with increased estrogen secretion, which leads to increased mammary gland growth in goats and sheep with twins. It was found that serum estradiol correlated positively with the number of fetuses in does (24). Furthermore, progesterone (PG) is an essential ovarian hormone needed for pregnancy's successful establishment and maintenance (22). Twin-bearing goats have an increased progesterone concentration (24). A measure of the amount of progesterone in sheep's blood can also determine the ovulation rate because the level of this hormone is lower in ewes that have ovulated once compared to those that have ovulated multiple times (22). Kakous *et al*. (25) observed higher steroid hormone levels in twins than in singletons. However, ovulatory rates and lambing rates are significantly increased with ewes able to produce more lambs per parturition with the enhancement of reproductive endocrinology and increased expression of oocyte growth factors (5). The oocyte-associated cumulus cells and litter size are controlled by coordinating sex hormone signals with oocyte-derived paracrine factors (BMP15) (7). The effect of the estradiol E2 signal depends on paracrine signals from oocytes and the synergistic effects of BMP15. The development of the cumulus cell and its function are enhanced by the coordinated estrogen signals and BMP15 (5). BMP15 is believed to be directly responsible for the proliferation of somatic cells and the regulation of steroidogenesis. The growth and survival of follicles are dependent on gonadotropin. BMP15 stimulates the proliferation of granulosa cells and modulates steroid hormone expression in these cells (7). Both estrogen receptors 1 and 2 (ER1 and ER2) play vital roles in estrogen metabolism in sheep ovaries, and they are positively correlated with BMP15 in large follicles. Ovarian function is also affected by the interactions between gonadotropins, estradiol, FSH, LH, and local ovarian factors like BMP15 (23).

Conclusion

The study identified a novel single nucleotide polymorphism (SNP) at c.50980646T>A in the *BMP15* gene. Individuals of the TA genotype had better levels of sex hormones and larger litters than those of the TT genotype. The litter size is controlled by coordinating sex hormone signals with oocyte-derived paracrine factors (BMP15). In light of these results, the c.50980646T>A mutation affects these traits positively. In this regard, *BMP15* polymorphisms can improve litter size in the livestock industry using marker-assisted selection programs.

Conflicts of interest

None.

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التغايرات الوراثية لجين بروتين مكون لشكل العظام وارتباطه بمستويات الأستروجين والبروجستيرون وعدد المواليد في الأغنام العواسي

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الخلاصة

هدف البحث لتقييم التباين الوراثي لجين بروتين مكون لشكل العظام فيما يتعلق بمستويات الهرمونات الجنسية ونوع الولادة في النعاج العواسي. تم عزل الحامض النووي منقوص الأوكسجين الجينومي لـ ١٣٨ نعجة عواسي (٥٢ نعاج تنتج ولادة مفردة و ٨٦ التي تنتج توائم). تم تضخيم جين بروتين مكون لشكل العظام الاكسون ٢ عن طريق تفاعل البلمرة المتسلسل. تم تحديد نوعين من الطرز الوراثية بناء على حجم القطعة ١٤١ زوج قاعدة: TT و TA. كشف تفاعل التسلسل الجيني عن طفرة جديدة، c.50980646T>A، في الطراز الوراثي TA. أظهر تعدد الأشكال للنيوكليوتيدات المفردة ارتباطا عاليا مع مستويات الهرمونات الجنسية وعدد المواليد، حيث كان لدى الأغنام التي تحتوي على هذه الطفرة مستويات أعلى من الهرمونات الجنسية وعدد مواليد أكبر من الأغنام بدونها. النعاج ذات الطراز الوراثي TA كان لديها ١,٨٩ من عدد المواليد أكثر من نظيراتها من TT. أكد الانحدار اللوجستي أن الطفرة c.50980646T>A زادت من عدد المواليد. في الاستنتاج، يبدو أن الطفرة c.50980646T>A مرتبطة معنويا بالصفات التناسلية (خاصة مستويات الهرمونات الجنسية وعدد المواليد) في الأغنام العواسي. مع هذه النتائج، فان طفرات جين بروتين مكون لشكل العظام مناسبة لتطوير برامج الانتقاء بمساعدة الواسمات لزيادة عدد المواليد في الأغنام العواسي.

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