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Novel single nucleotide polymorphism G216A of hormone-sensitive lipase gene associated with Awassi sheep reproduction

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Abstract

Background Litter size plays a crucial role in determining profitability in the sheep industry. Breeding sheep with high litter sizes could be enhanced by selecting candidate genes. One gene affecting sheep's reproductive performance is the hormone-sensitive lipase (*HSL*) gene. As a result, this study investigated whether the *HSL* gene variation influenced the fertility of Awassi ewes. The genomic DNA was extracted from 52 singleton ewes and 48 twin ewes. The *HSL* gene exon 9 (278 bp) was amplified using polymerase chain reaction (PCR).

Results Study results revealed two genotypes identified in the 278-bp amplicons: GG and GA. Molecular sequence analysis identified a novel mutation in the GA genotype 216G > A. The statistical analysis revealed a significant association between the single nucleotide polymorphism (SNP) 216A > G and reproductive performance. Ewes with the SNP 216G > A genotype exhibited significantly increased litter sizes, twinning rates, lambing rates, and fewer days to lambing compared to ewes with GG genotypes ($P \leq 0.05$). The logistic regression analysis results provided strong evidence that the 216G > A mutation significantly increased litter sizes.

Conclusions This study concluded that variant 216G > A SNP positively impacts Awassi sheep reproduction. There is a higher litter size and more prolificacy in ewes with the 216G > A SNP than in those without the SNP.

Keywords Adipose tissue, Fertility, *HSL* polymorphism, Sheep

1 Background

A sheep (*Ovis aries*) provides valuable agricultural products to people, making it an important economic animal. Increasing ewe productivity and reproductive performance generally enhances the economic and biological efficiency of the sheep industry [1, 2]. Therefore, identifying key genes influencing these traits is a crucial first step in developing a marker-assisted selection tool [3]. The identification of gene polymorphisms also holds great significance in the field of domestic animal breeding. It provides valuable insights into animal genotypes and their direct impact on reproduction, productivity, and overall economic efficiency. This understanding is essential for achieving remarkable advancements in the sheep industry [4]. Among the most crucial genes that affect energy intake and economic traits is the hormone-sensitive lipase (*HSL*) gene [5]. There are 14 exons in the ovine HSL gene located on chromosome 14 (GenBank Gene ID: 100169699). This gene encodes HSL, an intracellular enzyme in adipose tissue and hormone-producing organs, such as the adrenals and gonads. This enzyme hydrolyzes a wide range of substrates, including acylglycerol, cholesteryl esters, and steroids [6]. According to Kazala et al. [7], HSL activity is crucial in the mobilization and maintenance of triacylglycerol, fat content and reproductive capacity can be determined by it. Thus,

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animal producers are researching genetic alterations to determine the ones that lead to desired phenotypes.

Several genetic variations have been reported in livestock species, but few have been further elucidated. Wallace et al. [8] investigated the relationship between circulating lipids and *HSL* gene expression in perirenal adipose tissue concerning intrauterine growth restriction and gender, revealing differences in *HSL* gene expression between males and females. Steroid hormones can activate the enzyme, which enhances lipolysis. According to Xu et al. [9], hormone-sensitive lipases are expressed in the fat tails of Tan sheep. Sheep tail development resulted in a greater protein expression pattern for HSL. In the study conducted by Al-Thuwaini et al. [10], a fascinating discovery is made regarding the *HSL* gene. Specifically, five intriguing SNPs are identified in exon 2 and 9 (g.151C > A, g.198C > T, g.213G > C, g.226G > T, g.232A > C, respectively). A significant association has been found between these genetic variations and carcass traits. A missense mutation in the *HSL* exon 8 (g.16734G > A, g.16896A > G, g.17388G > T) strongly affected fat content in bovine intramuscular tissues [11]. According to Ibrahim [5], two SNPs in the *HSL* gene have been detected in Barki lambs (c.1865C > T and c.2038T > C). As a result, the *HSL* gene is an excellent candidate for selection by genetics and breeding. Until now, there have been few studies exploring the relationship between *HSL* polymorphisms and livestock reproduction traits. Sheep reproduction has not yet been studied with these polymorphisms. Consequently, this study investigated how genetic variations in the *HSL* gene influence Awassi sheep reproduction.

2 Methods

2.1 Sheep population
An ethical committee approved the study (Agri, No. 01, 7, 22) between July 2022 and September 2023. The study involved 100 Awassi ewes that were 3–4 years old, healthy, not pregnant or lactating, and sexually mature with weights ranging from 45 to 55 kg. The ewes reached puberty at 9 months of age and the parity of ewes was two to three parities. The ewes used in this study represent a subsample of available ewes selected from among the ewes that were pregnant or lactating. In the studied flock, 10–12 rams were randomly allocated to mate with approximately 20–25 ewes per ram, and all 3- to 4-year-old Awassi ewes from the entire flock were chosen. After ewes lambed, they were categorized as either twins (n = 48) or singletons (n = 52) based on the number of lambs they gave birth to (two for twins and one for singletons). Feeding included barley, wheat bran, and salt at precise ratios of 59%, 40%, and 1%, respectively. This carefully calculated mixture constituted 2.5% of their body weight. Three kilograms of alfalfa hay and one kilogram of straw were also fed to the animals. Animals were provided with fresh water throughout the day. Several prolific traits were recorded at the breeding stations, including twinning rate (twinning rate number of ewes with twins/number of ewes giving birth × 100), lambing rate (lambing rate lambed ewes/inseminated ewes × 100), survival rate (refers to the survival of the lambs born to genotyped ewes during the study at three months of age/number of lambs born × 100), number of days to lambing (the number of days between the joining of rams until the subsequent lambing), age at first lambing (the age of the ewe at her first lambing at about 1.5 years old) that was recorded from lambing records for the past 3- to 4-year-olds, and litter size (the number of lambs born per ewe lambing).

2.2 Extraction and amplification
Genetic testing was conducted on jugular vein blood samples collected from sheep. Genomic DNA was extracted from whole blood using the efficient salting-out method [12]. A total of 100 genetic sequences within the *HSL* gene were amplifiable using the NCBI Primer-BLAST program. The exact location of ovine *HSL* was determined using GenBank accession number NC_056067.1 (Fig. 1). The ovine *HSL* gene (exon 9) was amplified using a pair of designed primers. Primers used in this study had the following sequences: F:5′–CCAACT CCCTCAAGAGCCTG–3′, R:5′–TGAGTAGAGGGG CATCC ACA–3′. The amplification process was carried out over 30 cycles, starting with denaturation, annealing, and elongation at temperatures of 94 ℃, 60.4 ℃, and 72 ℃, respectively, each lasting 30 s. After a 5-min extension at 72 ℃, the sample was stored at 4 ℃ for further analysis [13]. Electrophoresis of a PCR product on a 2% agarose gel was conducted using a Gel Imager from Bio-Rad [14].

2.3 SSCP (single-strand conformation polymorphism) and DNA sequencing
Each PCR product was genotyped by Mohammed et al. [15]. SSCP analysis was performed using a 10 μL mixture of loading dye and each amplicon. Next, the mixture was heated to 95 ℃ for 7 min to denature it. Afterward, it was quickly cooled on ice for 10 min. Finally, the mixture was loaded onto 12% acrylamide: bisacrylamide (37.5:1; Bio-Rad) gels. The sample was electrophoresed for 4 h at 100 V and 200 mA in 1 × TBE buffer. Silver nitrate staining was used to visualize DNA fragments [16]. Sanger sequencing was employed to sequence all PCR products on polyacrylamide gels after detecting the SSCP samples. The sequencing results were analyzed using SnapGene Viewer (http://www.snapgene.com)/BioEdit
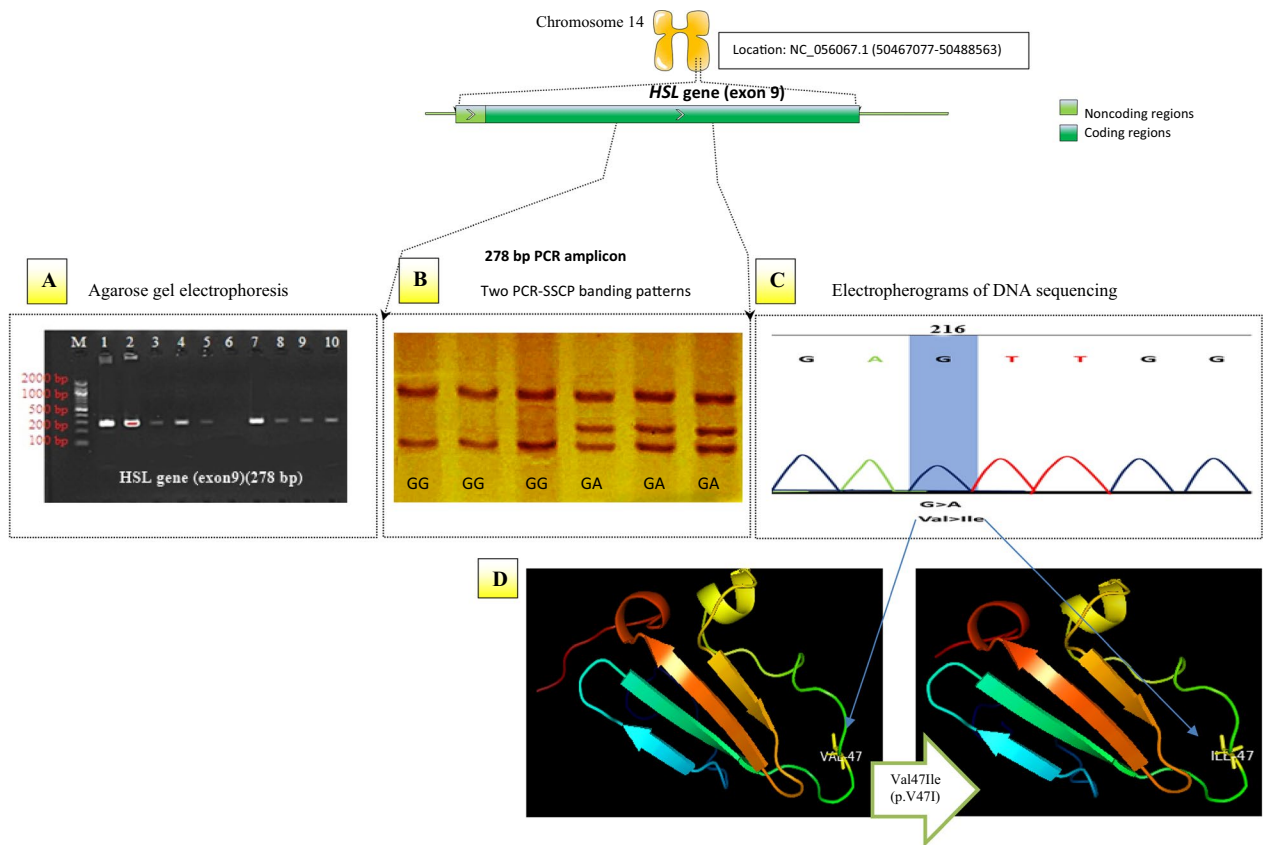
Fig. 1 An overview of the PCR-SSCP-sequencing technique applied to *HSL* gene analysis within Awassi ewes. **A** Agarose gel electrophoresis for PCR products of the exon 9 amplification. **B** PCR-SSCP genotyping, showing homozygous and heterozygous variations in exon 9. **C** Electropherograms of DNA sequencing were obtained for the GA genotype, which showed the SNP 216G > A in exon 9. **D** Characterization of the missense SNP, V47I, caused by the observed G216A SNP in the 3D structure of HSL, in which V47I was positioned before and after mutation

(DNASTAR, Madison, USA) to identify potential SNPs. A novelty assessment was conducted using Ensemble Genome Browser 96 (https://asia.ensembl.org/index.html). PyMOL version 7.0.1 (The PyMOL Molecular Graphics System, Schrödinger, LLC.) was used to create the 3D structure of HSL, and subsequent mutations were performed.

2.4 Data analysis

Software Popgene 1.31 was used to calculate genetic diversity [17]. A polymorphism information content (PIC) was calculated based on Botstein et al. [18]. The association analysis of *HSL* genotypes was conducted using IBM SPSS 23.0 (NY, USA) as follows:

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

where Y_{ijk} = phenotypic traits, μ = overall population mean, G_i = fixed effect of ith genotype (i = GG, GA), P_j = fixed effect of jth parity (j = 1, 2, 3), and e_{ijkl} = random residual error. The Bonferroni test determined a

significant difference at the significance levels of 0.05 and 0.01. Three reproductive traits (twinning rate, lambing rate, and survival rate) were analyzed using the Chi-square test [19]. An analysis of logistic regression was conducted on *HSL* polymorphisms and litter size. The lambing season, age, and factor interactions were removed from a multivariate analysis model if they were non-significant.

3 Results

3.1 Genetic analysis

As exon 9 was previously tested in Awassi sheep for growth traits [10], it was selected for HSL genotyping in the ewes included in this study. One genetic fragment of 278 bp was amplified from exon 9 and the flanking regions of the HSL gene (Fig. 1A). On a PCR-SSCP pattern designed for amplification of exon 9, two distinct patterns were observed (Fig. 1B). Sequencing analysis of the 278 bp amplicons confirmed that the SNP c.216G > A occurred only in one of the SSCP variants. The identified SSCP variants showed two genotypes: GG and GA,

Table 1 *HSL* gene diversity in Awassi ewes indicated by PCR-SSCP

Genotype frequencies (n)		Allele frequencies		Ho	He	Ne	PIC	χ^2/*P*-value
GG	GA	G	A					
0.58 (58)	0.42 (42)	0.79	0.21	0.42	0.33	1.49	0.28	6.87/**0.008**

n, number of individuals; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *Ne*, effective allele frequency; PIC, polymorphism information content; χ^2, Chi-square. All Chi-square tests have one degree of freedom and are within the significance level $P \le 0.05$

Table 2 The effect of *HSL* genotypes on reproductive traits in Awassi ewes

Genotypes	Number of lambs born	Birth type number (%)		Lambing rate (%)	Lamb survival rate %	Days to lambing (LSM ± SE)	Age at first lambing (LSM ± SE)
		Singleton	Twin				
GG (n = 58)	GG (n = 78)	38 (65.51%)	20 (34.48%)	51/58 (88%)	77 (98.71%)	167[b] ± 9.11	524.67 ± 23.48
GA (n = 42)	GA (n = 70)	14 (33.33%)	28 (66.66%)	41/42 (98%)	69 (98.57%)	158[a] ± 8.64	519.86 ± 27.14
P-value		**0.003**	**0.01**	**0.02**	0.52	**0.04**	0.34

LSM ± SE, Least square means ± Standard error, [a,b]Significant differences in means are represented by differences in the same column within each classification, the *P* value with statistical significance is indicated in bold numbers

evidenced by the presence of homozygous G/G patterns and heterozygous G/A patterns resulting from this nucleic acid substitution (Fig. 1C). Using Expasy software, it was determined that this SNP causes the substitution of valine for isoleucine at position 47 of the mature HSL protein (*p*. Val > Ile 47) (Fig. 1D).

Based on genetic diversity, Table 1 summarizes the results of the Hardy–Weinberg equilibrium, genotypes, and allele frequencies for Awassi sheep. In the Awassi populations, the Hardy–Weinberg equilibrium deviates, as demonstrated by the chi-square values at significance levels of $P \le 0.05$. Ovine HSL exhibited a moderate degree of polymorphism in the current analysis. Low, moderate, and high polymorphism were classified based on the PIC value. PIC values below 0.25 were classified as low, while those ranging from 0.25 to 0.5 were deemed medium, and above 0.5 was considered high.

3.2 Association analysis

The SNP c.216G > A did not reveal any significant difference ($P \le 0.01$) between GG and GA genotype individuals in survival rate and age at first lambing. A statistically significant association was found between GG genotypes and reduced litter sizes, twinning rates, lambing rates, and longer lambing days at the c.216G > A locus (Table 2). The logistic regression analysis presented in Table 3 sheds further light on the correlation between the c.216G > A mutation and litter size. According to Awassi data, GA genotype ewes had an average of 1.66 lambs, while GG genotype ewes had an average of 1.34 lambs. Thus, the SNP c.216G > A positively influenced these traits.

Table 3 Results of logistic analysis of the 216G > A SNP on litter size effects in Awassi ewes

Genotype	Litter size (LSM ± SE)	Logistic regression analysis		
		β	Odds ratio (95%CI)	*P*-value
GG	1.34[b] ± 0.16	1.00	Reference	**0.001**
GA	1.66[a] ± 0.19	1.09	2.97 (0.99–4.34)	

LSM ± SE, Least square means ± Standard error, β: Regression coefficients, [a,b] Significant differences in means represented by differences in the same column within each classification, the *P* value with statistical significance are indicated in bold numbers, CI: confidence interval

4 Discussion

The *HSL* gene variation has been identified in several animal studies. The two synonymous mutations identified by Zidi et al. [20] are located in goats (c.327C > A > T and c.558C > T in exon 2 and exon 3). A total of three SNPs (rs109598915, rs109759779, and rs41887406) are identified as part of the *HSL* gene in cattle by Goszczynski et al. [21]. The polymorphism of the *HSL* gene (c.276T > C and c.219C > A in exon 2 and exon 6) has been observed in Holstein–Friesian cows [22]. According to Pe'iulaitien et al. [23], RFLP-PCR is also used to identify the c.442 G > A mutation in the HSL gene in hybrid pigs.

An SNP in exon 9 of HSL is also identified by Ibrahim [5] using PCR-SSCP in Barki sheep (c.1865C > T and c.2038T > C). Based on polymorphisms in intron 4 of the HSL gene, Kong et al. [24] identified a novel SNP (g.4819 A > G) in Hu sheep. Despite this, there is limited literature on variations in *HSL* among Awassi sheep. Many Middle Eastern countries have the Awassi breed of sheep [25]. Although this breed is known for its resistance to

adverse environments, they are not as prolific as Karakuls and Assafs [1]. A breed with low reproductive capacity concerns breeders in the Middle East, prompting them to make efforts to enhance reproductive capacity. The discovery of polymorphism in the *HSL* gene represents an exciting opportunity to enhance litter size in the Awassi ewe population.

Regarding association results, the SNP c.216G > A in the *HSL* gene was linked with Awassi sheep reproductive traits. As indicated by the statistical analysis, there was a significant association ($P \leq 0.01$) between the c.216G > A mutation and prolificacy. GA genotypes exhibited increased litter sizes, twinning rates, lambing rates, and shorter lambing periods than GG genotypes. Consequently, the c.216G > A mutation positively affected these traits. The cause may be that HSL hydrolyzes a wide range of substrates, including acylglycerol, diacylglycerol, and monoacylglycerol, to play a role in metabolism, homeostasis, and steroidogenesis, thus affecting reproductive hormones [5]. Reproductive hormones depend on lipid metabolism for the development of follicles and the maturation of oocytes [26]. Their vital role in reproduction is reflected in the high-density lipid accumulation [27]. The collaboration between HSL, adipose triglyceride lipase, and monoacylglycerol lipase has a synergistic effect on the breakdown of fats in adipocyte lipid droplets, converting triacylglycerol into non-esterified fatty acids [22]. Recent research has shown that functional fatty acids stimulate steroidogenesis and lipid metabolism in granulosa cells in livestock, thereby enhancing follicular growth and oocyte quality [26]. Furthermore, HSL is an intracellular neutral lipase enzyme encoded by the *HSL* gene, therefore, sequence variations in its structure could potentially modify the fatty acid composition in tissues and affect economic traits [10]. Genetic improvements in economic traits such as litter size are the major focus of the sheep breeding program [1]. HSL gene has the potential to be a valuable tool due to its impact on economic traits. Despite the studies mentioned above, HSL genotypes have not been investigated in Awassi sheep. This study enhances our understanding of genotypes and identifies new associations that could be valuable for identifying sheep with improved reproductive traits through marker-assisted selection programs.

5 Conclusions

A novel SNP, 216A > G, was identified in the heterozygous GA genotype. Ewes possessing the GA genotype displayed increased litter sizes, elevated twinning rates, enhanced lambing rates, and reduced time intervals between lambing when compared to ewes with the GG genotype. These results indicate that ewes carrying the 216A > G SNP are more prolific and conceive lambs. The results of this study could also be applied to marker-assisted selection programs to enhance the reproductive performance of sheep. Further studies involving a larger number of Awassi ewes are needed to elucidate the relationship between polymorphisms in the HSL gene sequence and reproductive traits.

Declarations

Ethics approvals and consent to participate
Research conducted on animals Al-Qasim Green University between July 2020 and March 2021 according to international guidelines for animal care and use, with approval number (Agri, No. 01, 7, 22).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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