Reproduction of Awassi and Hamdani Sheep Is Associated With a Novel Missense SNP (p.24Ile>Thr) of the *GnIH* Gene

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ABSTRACT

OBJECTIVES: Litter size is a crucial economic factor in the sheep industry. Several factors and genes influence litter size, making the identification of genes or loci involved a genetic challenge. Gonadotropin-inhibitory hormone (*GnIH*) is one of several genes that influence sheep's reproductive traits. Thus, this study aimed to investigate whether variations in the *GnIH* gene affect the reproductive performance of Awassi and Hamdani ewes.

METHODS: DNA was extracted from 99 single-progeny ewes and 101 twin ewes. The polymerase chain reaction (PCR) produced amplicons of 262 bp, 275 bp, and 284 bp from exons 1, 2, and 3 of the *GnIH* gene. Single-strand conformational polymorphism (SSCP) technique was used for genotyping experiments. Sequencing and in silico analysis were performed on each set of SSCP-resolved bands.

RESULTS: Two genotypes of 262 bp amplicons were found: TT and TC. Sequence analysis revealed a novel missense mutation in the TC genotype at position c.122T>C. Five in silico tools were used to assess the impact of this mutation on GnIH protein structure, function, and stability, all of them demonstrated a deleterious effect. An analysis of statistical data revealed a strong correlation between the c.122T>C single-nucleotide polymorphism (SNP) and reproductive performance. Ewes with the SNP 122T>C exhibited a significant increase in litter size, twinning rates, lambing rates, and days to lambing when compared with ewes with the TT genotype. A lower number of lambs were born to ewes with the TT genotype than those with the TC genotype.

CONCLUSION: These results concluded that the c.122T>C SNP variant positively influences the reproductive performance of Awassi and Hamdani sheep. Sheep that carry the c.122T>C SNP show higher litter size and increased productivity.

KEYWORDS: In silico, *GnIH* polymorphism, twinning rate, sheep

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Introduction

Sheep reproduction is commonly known to be influenced by distinct seasonal patterns, which poses significant challenges for improving sheep farming.[1] Sheep farming is generally more efficient and profitable when the productivity and reproduction of ewes are improved.[2-4] Numerous genetic loci associated with economic valuable traits in farm animals have been identified through advancements in molecular genetics. The development of marker-assisted selection tools begins with identifying the candidate genes that influence these traits.[5] The gonadotropin-inhibitory hormone (*GnIH*) gene is a promising candidate for polymorphism analysis related to reproductive performance.[6] The *GnIH* gene consists of 3 exons on sheep's chromosome 4 (NCBI Reference Sequence NC_056057.1) and goat's chromosome 4, encoding the GnIH hormone.[6] The hypothalamic neuropeptide hormone GnIH plays a crucial role in inhibiting the production of reproductive hormones by competing with the gonadotropin-releasing hormone (GnRH).[7] Furthermore, GnIH effectively reduces cAMP production and GnRH-induced phosphorylation of extracellular signal-regulated kinase.[8] Gonadotropin-inhibitory hormone neurons also express estrogen receptors, indicating their involvement in the negative feedback loop between estrogen and GnRH that regulates reproductive activity.[9] Administration of GnIH to ewes resulted in a significant reduction in estrogen-induced luteinizing hormone (LH) surges. Ewes in the preovulatory stage also experience a decrease in gene expression of *GnIH*. The surge of GnRH/LH in ewes may also be induced by a decrease in GnIH.[10] Through its control over these hormones, GnIH contributes to the regulation of reproductive traits.[11] Gonadotropin-inhibitory hormone plays a significant role in the secretion of gonadotropins in both avian and mammalian species.[12] As a result, variations in the *GnIH* gene have a significant impact on biological traits.

Numerous studies have shown that livestock productivity is strongly related to single-nucleotide polymorphisms (SNPs) in key regulatory genes.[13,14] Until now, only one study has been found to demonstrate that *GnIH* plays a role in regulating litter size. Two variations, g.1837C>G and g.3195G>A, have been identified in the *GnIH* gene, which is associated with litter size in dairy goats. Genetic associations suggest that individuals with the CC/GG genotype of 2 SNPs have a significantly

Table 1. The oligonucleotide primer sets are designed for the amplification of the ovine *GnIH* gene.

PRIMER CODE	LOCUS	SEQUENCE (5′-3′)	BINDING COORDINATES IN THE GENOME		AMPLICON LENGTH	ANNEALING TEMPERATURE
			START	STOP		
GnIH, exo1-F	Exon 1	TGTATAGACATCAGGCTGCACA	72260451	72260472	262 bp	60.9 °C
GnIH, exo1-R		ACAAAAGCACTAGACTCAGAACA	72260712	72260690		
GnIH, exo2-F	Exon 2	CTGCCGTCAACAAAATGCCA	72262131	72262150	275 bp	55.3 °C
GnIH, exo2-R		CCGGCAGGTCATGGAGTAAA	72262405	72262386		
GnIH, exo3-F	Exon 3	GTGGCAATGAGGCAGAGAGAT	72263560	72263580	284 bp	56.4 °C
GnIH, exo3-R		AGGGACAGGCTCCACATTTC	72263843	72263824		

The symbols "F" and "R" refer to forward and reverse primers, respectively. The design was based on the ovine NCBI Reference Sequence NC_056057.1.

larger litter size than those with GG/AA genotypes.[6] As a result, the *GnIH* gene is an excellent candidate for genetic selection and breeding. However, the *GnIH* gene polymorphism has not yet been studied in terms of its impact on the reproductive performance of Awassi and Hamdani sheep. Thus, the study fills this knowledge gap by identifying polymorphisms in the *GnIH* gene and evaluating their impact on reproductive performance in Awassi and Hamdani sheep. This study used state-of-the-art in silico tools to investigate the genetic diversity and polymorphism of the *GnIH* gene in the study population and to assess whether the mutation affects GnIH protein structure, function, and stability. After that, *GnIH* genotypes of Awassi ewes were analyzed for litter size, lambing rate, number of days to lambing, and age at first lambing. Furthermore, the study examined the relationship between litter size and *GnIH* variants.

Materials and Methods

Sheep population

The study was approved by the Al-Qasim Green University Animal Ethics Committee in compliance with international guidelines (Agri, No. 01, 7, 22). Awassi (130) and Hamdani (70) ewes were selected for this study. The Awassi and Hamdani are the most prevalent breeds among those in the Middle East. Awassi sheep weigh 40 to 45 kg, while Hamadani sheep weigh 55 to 60 kg. The 2 animal breeds differed in terms of genetics and geography. Despite this, they are characterized by reproductive seasonality and have lower reproduction rates than Karakuls and Assafs. Thus, breeding improvements in Awassi and Hamadani sheep may be achievable through the *GnIH* gene. Ewes were sexually mature, healthy, and 3 to 4 years old, and classified into 99 ewes-producing singletons and 101 ewes-producing twins. Animals were randomly selected from each breed at Babylon and Karbala stations. All animals were under the same conditions of management, feeding, and weather. In the breeding stations, several prolific traits were recorded, including twinning rate (the propensity to have more twin litters per 100 ewes at the same average lambing percentage), lambing rate, 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), and litter size (the number of lambs born per ewe lambing).

Molecular analysis

DNA was extracted from whole sheep blood using a rapid salting-out technique.[15] The NCBI Primer-BLAST server was used to design 3 genetic fragments that cover the coding regions of the *GnIH* gene. Using NCBI Primer-BLAST, 200 genetic sequences were amplified from the genetic material within *GnIH*.[16] The Bioneer premix and Eppendorf thermal gradient apparatus were used to identify the optimal polymerase chain reaction (PCR) amplification conditions, as indicated in Table 1. The process involved an initial denaturation step at 94 °C for 5 minutes, followed by 30 cycles of 30 seconds of denaturation, 45 seconds of annealing, and 30 seconds of extension. A final extension was performed at 72 °C for 5 minutes, and then the samples were stored at 4 °C.[17] The quality of the PCR products was assessed through electrophoresis in 2% agarose gels, and images were taken using a Chemidoc Gel Imager (Bio-Rad, Hercules, CA, USA).[18]

In light of the high accuracy of PCR single-strand conformation polymorphism (SSCP) for both the detection of known mutations and unknown mutations in PCR amplicons, a recently recommended rapid PCR-SSCP genotyping protocol was used. A genotype was determined for each PCR product using the technique described by Mohammed et al.[19] The PCR product (10 μL) was mixed with 10 μL of SSCP loading buffer dye (Bio-Rad). The PCR/SSCP products were initially subjected to 95 °C for 7 minutes. Afterward, they were promptly cooled on ice for 10 minutes before undergoing separation on a non-denaturing polyacrylamide gel.[20] Electrophoresis was conducted in a vertical unit using a 1× TBE (Tris boric EDTA) buffer. A 10% SSCP gel was prepared using a mixture of acrylamide and bisacrylamide at a ratio of 37.5:1.[21] The gel was electrophoresed at a constant current of 200 mA and a voltage of 100 V for 4 hours, at a running temperature of 4 °C. Silver

nitrate was used as a staining agent in the gels, as described by Byun et al.[22] A gel documentation system was used to photograph and analyze heterozygous and homozygous genotypes exhibiting different SSCP patterns. Polyacrylamide gel bands were detected using SSCP, followed by Sanger sequencing of all PCR products. BioEdit 7.1 and SnapGene Viewer 4.0.4 were used for analyzing the sequencing results. Based on the Ensemble Genome Browser 96 novelty detection, the observed variants were classified as novel. The ExPASy software was used to detect amino acid reading frames.[23] The next step involved comparing the amino acid sequences with those in the GnIH database using UniProtKB (http://www.uniprot.org/align/). The structure, function, and stability of mutant proteins were predicted using a variety of computational tools, including Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, Provean, I-Mutant2, and Mupro.

Data analysis

The genotypes and allele frequencies, observed (Ho) and expected (He) heterozygosities, and effective number of alleles (Ne) were calculated using the PopGen32 program, version 1.31.[24] The populations were assessed for Hardy-Weinberg equilibrium (HWE) using a chi-square test, as well as for polymorphism information content (PIC) based on the methodology outlined by Botstein et al.[25] $PIC = 1 - \sum_{i=1}^{m} p_i^2 - \sum_{i=1}^{m-1} \sum_{j=i+1}^{m} 2 p_i^2 p_j^2$, where p_i and p_j are the frequencies of the ith and jth alleles, respectively, and m is the number of alleles.

The association analysis of *GnIH* genotypes was conducted using IBM SPSS 23.0 (NY, USA) as follows:

$$Y_{ijkl} = \mu + G_i + B_j + P_j + e_{ijkl}$$

where $Yijkl$ = phenotype characteristics, μ = the mean of all traits, G_i = fixed effect of the ith genotype (i = TT, TC), B_j = fixed effect of the jth breed (j = 1, 2), Pk = fixed effect of the kth parity (k = 1, 2, 3), and e_{ijkl} = random error. The Tukey-Kramer test was used to determine whether there was a significant difference at the 0.05 and 0.01 levels of significance. Three reproductive traits were assessed using the chi-square test: lambing rate, survival rate, and litter size. Factor interactions, lambing season, and age, and their effects were excluded when they were not significant. The in silico tools SIFT, PolyPhen-2, PROVEAN, I-Mutant2.0, and Mupro were used to assess the potential damaging or non-damaging impacts of the identified missense variant on the GnIH protein's structure, functions, and stability.

Results

Genotyping, sequencing of GnIH genes, and genetic diversity

All coding regions of the *GnIH* gene were screened by amplifying exon 1, exon 2, and exon 3 with PCR fragments of 262, 275, and 284 bp, respectively (Figure 1A). In the 275 bp and 284 bp amplicons, electrophoretic migrations showed monomorphism for the amplicons corresponding to exons 2 and 3. The 262 bp amplicons intended for amplification of exon 1 exhibited 2 distinct patterns of PCR-SSCP (Figure 1B). Sequencing experiments confirmed that c.122T>C occurred only in one variant of exon 1, indicating heterogeneity. As a result of the detected c.122T>C nucleic acid substitution, TT and TC genotypes were identified, corresponding to the homozygous T/T and heterozygous T/C patterns observed in the coding sequence of exon-1 amplicons, respectively (Figure 1C). Expasy-translate analysis showed that this nucleic acid substitution causes isoleucine to threonine substitution at position 24 of the mature GnIH protein (p.Ile>Thr24). This substitution was investigated using 5 computational tools: SIFT, PolyPhen-2, Provean, I-Mutant2, and Mupro to assess the potential deleterious or neutral consequences of this missense SNP on the structure, function, and stability of the protein. According to all computational analyses (Figure 1D), the effects were deleterious and reduced stability. In contrast to individuals with TT genotypes, individuals with TC genotypes exhibited adverse effects of this nsSNP on the scheduled biological activities of GnIH protein.

Regarding genetic diversity, the results of HWE, genotypes, and allele frequencies for Awassi and Hamdani sheep are presented in Table 2. In a genetic diversity analysis of the *GnIH* gene, the genotype TT was the most frequent (n = 79 in Awassi and n = 41 in Hamadani). The TC genotype was prevalent at 0.39 (n = 51) in Awassi sheep and at 0.41 (n = 29) in Hamadani sheep. According to polymorphic information content (PIC) analysis, values below 0.25 were considered low, those between 0.25 and 0.5 were considered medium, and those above 0.5 were considered high. The c.122T>C locus showed a moderate level of polymorphism. A chi-square test revealed that the c.122T>C SNP significantly deviated from HWE in both the Awassi and Hamdani populations, with significance levels of $P \leqslant .05$.

Association analysis

In an association analysis, the c.122T>C SNP did not result in a significant difference ($P \geqslant .01$) in survival rate between TT and TC genotyped ewes. At the same c.122T>C locus, the TC genotype was significantly associated ($P \leqslant .01$) with larger litter size, higher twinning rates, increased lambing rates, and fewer days of lambing (Table 3). Compared with the TT genotype, the TC genotype produced 1.69 lambs per ewe in Awassi and 1.59 lambs per ewe in Hamdani. This observation indicated a potential association between ewes with the TC genotype and a higher litter size. Consequently, these traits were positively correlated with the SNP c.122T>C.

Discussion

Genomic tools are truly remarkable because they reveal the specific genes that are crucial in influencing a wide range of reproductive traits. This capability enables us to achieve

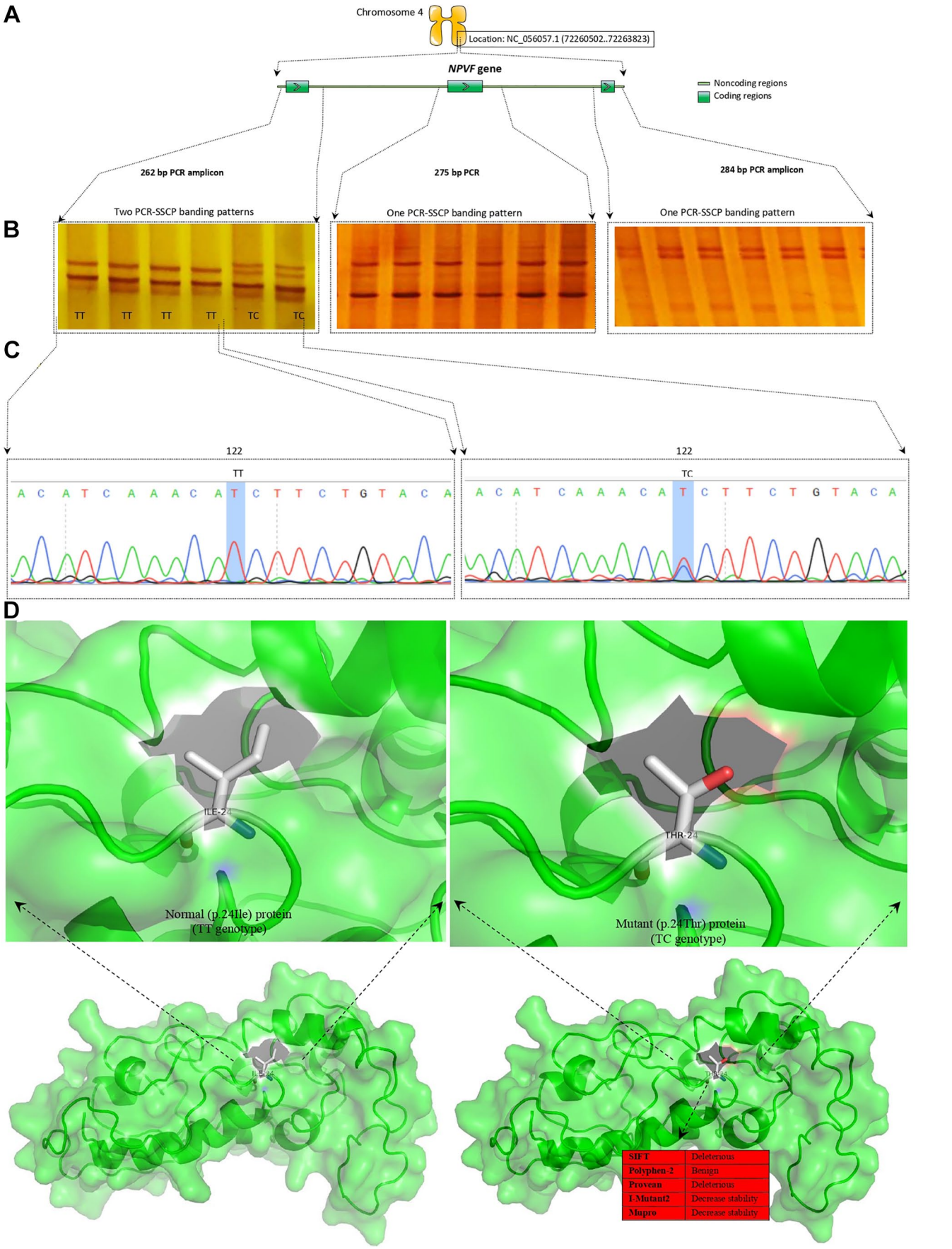

Figure 1. An illustration of the PCR-SSCP-sequencing technique using the *GnIH* gene in Awassi ewes. (**A**) PCR-specific primers were designed to amplify 262 bp, 275 bp, and 284 bp in exon 1, exon 2, and exon 3, respectively. (**B**) PCR-SSCP genotyping confirmed homozygosity and heterozygosity in exon 1. (**C**) Electropherograms of DNA sequencing reveal a c.122T>C SNP in exon 1 of the TT genotype. (**D**) Implementation of 5 bioinformatics techniques to study the deleterious effects of the p.Ile>Thr24 SNP on the *GnIH* protein.

Table 2. Genetic diversity of the *GnIH* gene in Awassi and Hamdani ewes detected by PCR-SSCP.

BREED	GENOTYPE FREQUENCIES (N)		ALLELE FREQUENCIES		*HO*	*HE*	*NE*	χ^2	*PIC*
	TT	TC	T	C					
Awassi	0.61 (79)	0.39 (51)	0.80	0.20	0.39	0.32	1.46	7.56	0.27
Hamdani	0.58 (41)	0.41 (29)	0.79	0.21	0.41	0.33	1.49	4.58	0.28

Abbreviations: χ^2, chi-square; *He*, expected heterozygosity; *Ho*, observed heterozygosity; n, number of individuals; *Ne*, effective allele frequency; PIC, polymorphism information content.
All chi-square tests have 1 degree of freedom and are within the significance level $P \leqslant .05$.

Table 3. The association between *GnIH* genetic polymorphism and reproductive performance in Awassi and Hamdani ewes.

BREED	GENOTYPES	BIRTH TYPE (%)		LAMBING RATE (%)	SURVIVAL RATE %	DAYS TO LAMBING (LSM ± SE)	LITTER SIZE (LSM ± SE)
		SINGLETON	TWIN				
A) Awassi	TT	41 (51.89%)	38 (48.10%)	96	116 (99.14%)	$170^{b} \pm 7.58$	$1.48^{b} \pm 0.11$
	TC	16 (31.37%)	35 (68.62%)	84	85 (98.83%)	$161^{a} \pm 9.21$	$1.69^{a} \pm 0.19$
	P-value	**.001**	**.002**	**.02**	.51	**.02**	**.03**
B) Hamdani	TT	30 (73.17%)	11 (26.82%)	93	51 (98.07%)	$172^{b} \pm 8.33$	$1.27^{b} \pm 0.20$
	TC	12 (41.37%)	17 (58.62%)	81	45 (97.82%)	$163^{a} \pm 7.41$	$1.59^{a} \pm 0.11$
	P-value	**.003**	**.001**	**.03**	.30	**.04**	**.006**

Abbreviation: 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.

significant genetic advancements that will last.[26] There are many genetic variants associated with litter size in sheep, such as the c.382C>A variant of the *BMP2* gene,[27] the g.22645332G>T variant of the *PTX3* gene,[28] as well as the rs399534524 locus in *CLSTN2* and the rs407142552 locus in *CTH*.[29] According to Li et al,[30] the variants c.1057-4C>T in *NOX4* and c.1983C>T in *PDE11A* are significantly associated with sheep litter size. Moreover, several loci have been associated with litter size in sheep, including the g.4054427728A>G *HRG* locus and the g.421655951C>T *FETUB* locus.[31] Tao et al[32] also found that *WWC2* (g.14962207C>T), *SLK* (g.27108855G>A), *ARHGEF9* (g.48271079), and *FSHR* (g.80789180T>G) affected sheep litter size. The above findings indicate the crucial role of *genetic variation* in influencing livestock reproduction. However, only one study has shown that the *GnIH* gene exhibits genetic variability in livestock reproduction. Two *GnIH* gene variants, g.C1837G and g.G3195A, have been identified in 2 breeds of Chinese dairy goats that are associated with litter size. These polymorphisms are identified through DNA sequencing and restriction fragment length polymorphism analysis.[6] In sheep, there is no information available regarding the role of *GnIH* in controlling litter size, and no research has been done on the variation of *GnIH* in Awassi and Hamdani sheep. Awassi and Hamdani breeds are widespread in Middle Eastern countries.[33,34] There are differences among breeds based on their morphological characteristics. The external environment and specific living conditions can affect sequence variation in Awassi and Hamdani breeds.[35,36] The Awassi sheep are renowned for their ability to adapt to adverse conditions,[37] while the Hamdani sheep originated in Iraq, where they flourish in the natural environment, yielding abundant amounts of meat, wool, and milk.[33] However, their reproductive rates are lower than those of Karakul and Assaf.[36] Therefore, the present study fills a knowledge gap by presenting new genotypic data and association findings.

This study found that the SNP c.122T>C located in the *GnIH* gene was associated with reproductive traits in Awassi and Hamdani sheep. Statistical analysis revealed that there is a significant association ($P \leqslant .01$) between the c.122T>C mutation and prolificacy, as the TC genotype showed higher litter size, twinning rate, lambing rate, and fewer days to lambing compared with the TT genotypes. Therefore, the c.122T>C SNP mutation had a positive effect on these traits. This may be due to that GnIH reduces sheep and cattle LH and follicle-stimulating hormone (FSH) production by directly suppressing GnRH.[6] In addition, GnIH regulates calcium flux, synthesis of gonadotropin subunits, and secretion of gonadotropins in

pituitary gonadotropes both in vitro and in vivo, showing that it is a peptide that negatively affects sheep reproduction.[38,39] The gonads, brain, and pituitary are the primary targets of GnIH and its receptor.[40] Furthermore, follicle granulosa cells express the GnIH and GnIH receptors, indicating that GnIH is involved in the production of gonadal steroid hormones and the maturation of germ cells through paracrine or autocrine mechanisms. Estradiol and progesterone are synthesized by granulosa cells under FSH and LH stimulation to facilitate follicle maturation and ovulation. As a result, GnIH inhibits folliculogenesis at granulosa cells of the porcine ovary and reduces central signaling *pathways* including cyclin-B1 levels and extracellular signal-regulated kinase 1/2.[41] Any detrimental effect on this inhibitor could therefore have a positive effect on reproduction. In this study, one missense SNP (p.Ile>Thr24) was discovered with a notably higher frequency in twin ewes genotyped from a 262 bp amplicon. A missense variant results in amino acid substitutions that could be tolerated, partially acceptable, or deleterious for protein function. As a result of this direct impact, freely available computational tools are increasingly enabling the prediction of missense variants.[42] Based on these considerations, numerous computer-based analyses confirmed the detrimental effects of the missense p.Ile>Thr24 SNP on the structure, biological functions, and stability of GnIH, which adversely affects its ability to perform its function. Consequently, the higher frequency of p.Ile>Thr24 results in damaged GnIH synthesis, leading to increased prolificacy in Awassi and Hamdani ewes. This study found that the *GnIH* gene may affect the reproductive performance of sheep breeds. The polymorphisms of the *GnIH* gene make it a promising candidate for marker-assisted selection, with the potential to significantly increase the litter sizes of ewes in the future. However, further studies with a larger sample size and more extensive records are needed. In addition, genome-wide association studies of Awassi and Hamdani sheep are expected to uncover genetic markers associated with litter size. This gene also requires further research in other sheep breeds to identify more polymorphisms, compare gene sequencing, and trace the evolutionary relationships among sheep breeds.

Conclusions

The *GnIH* gene of Awassi and Hamdani sheep breeds had a significant impact on litter size when the SNP c.122T>C was present. The TC genotype was linked to increased litter sizes, higher twinning rates, improved lambing rates, and a shorter time to lambing compared with the TT genotype in ewes. Individuals with TC genotypes showed better reproductive traits compared with those with TT genotypes. Marker-assisted selection makes it possible to choose individuals with increased litter sizes and high prolific qualities in both sheep breeds. Selecting sheep with TC genotypes and conducting future studies with larger sample sizes and more sophisticated techniques to determine whether this gene is associated with high prolificacy is crucial.

Author Contributions

Both authors contributed equally. In addition, both authors reviewed and approved the final manuscript.

Ethical Approval

This research was approved by Al-Qasim Green University's research committee and was conducted according to the international guidelines on animal care and use (Agri, No. 01, 7, 22).

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Data Availability Statement

All data generated or analyzed during this study are included in this manuscript and its information files.

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