Association of Novel C319T Variant of *PITX2* Gene 3'UTR Region With Reproductive Performance in Awassi Sheep

Bioinformatics and Biology Insights
Volume 17: 1–7

DOI: 10.1177/11779322231179018

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ABSTRACT: Several genes influence sheep's reproductive performance, among them the paired-like homeodomain transcription factor 2 (*PITX2*) gene. Thus, this study aimed to examine whether the variability within the *PITX2* gene is associated with the reproductive performance of Awassi ewes. A total of 123 single-progeny ewes and 109 twin ewes were used to extract genomic DNA. An amplicon of 4 sequence fragments from exons 2, 4, 5 (upstream portion), and 5 (downstream portion) of the *PITX2* gene was generated by polymerase chain reaction (PCR), 228, 304, 381, and 382 bp, respectively. Three genotypes of 382 bp amplicons were identified: CC, CT, and TT. Sequence analysis revealed a novel mutation in the CT genotype 319C > T. Statistical analysis revealed that single-nucleotide polymorphism (SNP) 319C > T was associated with reproductive performance. Single-nucleotide polymorphism 319C > T-carrying ewes had significantly ($P \leq .01$) lower litter sizes, twinning rates, lambing rates, and more days to lambing than those carrying CT and CC genotypes. Based on a logistic regression analysis, it was confirmed that the 319C > T SNP decreased litter size. Ewes with TT genotype produced fewer lambs than ewes with CT and CC genotypes. According to these results, the variant 319C > T SNP negatively affects the reproductive performance of Awassi sheep. Ewes carrying the 319C > T SNP have a lower litter size and are less prolific than those without the SNP.

KEYWORDS: Fertility, litter size, RIEG, polymorphism, sheep

RECEIVED: November 13, 2022. **ACCEPTED:** May 13, 2023.

TYPE: Original Research Article

FUNDING: The author(s) received no financial support for the research, authorship, and/or publication of this article.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Reproductive performance is among the most important features associated with sheep[1,2] that is potentially affected by genetics and the environment.[3] Identifying the genetic mechanisms responsible for lamb reproductive performance is imperative to identify the candidate genes and their causal mutations.[4,5] The gremlin 1 (*GREM1*) gene,[6] the prolactin gene,[7] and the oxidized low density lipoprotein receptor 1 (*OLR1*) gene[8] have all been found to stimulate reproductive function in Awassi sheep. Another gene, paired-like homeodomain transcription factor 2 (*PITX2*), has also been found to play a role in livestock reproduction.[9] The *PITX2* gene resides on chromosome 6 in sheep and contains 6 exons (adopted from ncbi.nlm.nih.gov), as well as on chromosome 6 in cows with 7 exons.[10] The *PITX2* gene regulates asymmetric organ development during animal development. Therefore, this gene is crucial to animal physiological function.[9] It has also been found that the *PITX2* gene is vital for myogenesis and muscle formation[10] and can control redox conditions and regulate the transcription factor myogenic differentiation (MyoD) during fetal and adult development.[11] *PITX2*, also known as *RIEG*, influences hematopoiesis, differentiation, and organogenesis.[12,13] *PITX2* expression results in the expression of all genes in our asymmetry marker panel, including *ERα*, which may contribute to sexual differentiation. Paired-like homeodomain transcription factor 2 also controls the bone morphogenetic protein 7 (*BMP7*) gene, which is present in the left gonad at the beginning of its development.[14] Many signal pathways require the *PITX2* gene, such as the Wnt/β-catenin pathway and the POU1F1-PROP1 pathway and can mediate mRNA stabilization in these pathways.[9] The Wnt/β-catenin signal pathway is crucial during embryonic development, development decisions, and tissue homeostasis of adult tissues.[15] In this way, PITX2 regulates gene expression by interacting with Wnt and growth factor pathways in cell types that are specifically responsive to this receptor.[16] Furthermore, PITX2 participates in the transcription and expression of genes involved in the POU1F1-PROP1 signal pathway, most notably *POU1F1*, *LHX3*, and *PROP1*, which contribute to growth hormone, prolactin, and reproductive hormone secretion.[17] The *PITX2* gene may affect animals because these hormones are crucial for their development, growth, and reproduction.[18] In addition, the *PITX2* is involved in signal transduction pathways that could influence reproductive processes.[19] This gene encodes the bicoid-like transcription factor PITX2 protein, which contains the homeobox-2 and the OAR domains. Paired-like homeodomain transcription factor 2 interacts with pituitary homeobox 1 through its OAR domain as well as its homeobox-2 domain. This is because the homeobox-2 domain has a helix-turn-helix (HTH) structure that can combine specifically with DNA.[20] The integrity of the homeodomain is essential for PITX2 to bind DNA.[21]

Table 1. The oligonucleotide primer sets designed for the amplification of the ovine *PITX2* gene.

PRIMER CODE	LOCUS	SEQUENCE (5'-3')	BINDING COORDINATE IN THE GENOME		AMPLICON LENGTH	ANNEALING TEMPERATURE
			Start	Stop		
PITX2,exo2-F	Exon 2	TTGAAGTCGTCTGCCCCACA	15196256	15196275	228 bp	59.8°C
PITX2,exo2-R		CCATAAGACCAGTGCCCTCTC	15196483	15196463		
PITX2,exo3-F	Exon 3	GATACTTTCCCCCGCCGTC	15196802	15196820	249 bp	NA[a]
PITX2,exo3-R		GAGGCAGGCGCCCAG	15197050	15197036		
PITX2,exo4-F	Exon 4	CGGGGACCTCTGTGTTTCG	15208134	15208152	304 bp	61.0°C
PITX2,exo4-R		TTTCACCCTCCCTCCTGATCT	15208437	15208417		
PITX2,exo5,1-F	Exon 5,1	CACTTATGTGTCGGAGGGGG	15210690	15210709	381 bp	59.8°C
PITX2,exo5,1-R		GACGACATGCTCATGGACGA	15211070	15211051		
PITX2,exo5,2-F	Exon 5,2	GCCCAATTCCATCTCGTCCA	15211038	15211057	382 bp	59.8°C
PITX2,exo5,2-R		CCCAGTCTTTCAAGGGCAGA	15211419	15211400		

The symbols "F" and "R" refer to forward and reverse primers, respectively. The design was based on the ovine NCBI Reference Sequence NC_056059.1.
[a]No specific results were obtained from utilizing this primers' pair to amplify this locus as no specific bands (~249 bp) were observed in agarose gel electrophoresis.

In several research studies, *PITX2* polymorphism has been related to phenotypic characteristics in livestock. According to Zhao et al,[12] variants in the *PITX2* gene influence milk quality in dairy goats and meat quality and growth traits in pigs.[22] In addition, the black-bone chickens of Wuliang Mountain have significant body size and carcass traits, which vary with polymorphisms in exons 1 and 3 of the *PITX2* gene.[20] In reproduction, *PITX2* has been shown to enhance twinning rates in chickens[18] because of its predominance in embryonic axes formation. Furthermore, Shaanbei white cashmere goats with a 22-bp indel mutation in the *PITX2* gene have significant growth and litter size.[9] The *PITX2* gene is, therefore, suitable for use in genetics and breeding as a candidate gene for marker-assisted selection. However, genetic polymorphisms in *PITX2* have only been studied in limited current studies on their effects on livestock reproductive traits, and to the researcher's knowledge, no research has been done on their impact on Awassi sheep reproductive performance. Thus, this study aimed to determine whether genetic variations in the *PITX2* gene affect reproductive performance in Awassi sheep.

Materials and Methods

Sheep population

The research was approved by the animal ethical committee of Al-Qasim Green University and performed from July 2021 and April 2022 according to international guidelines on animal care and use (Agri, No. 015, 7,20). A total of 232 sexually mature ewes between 3 and 4 years of age were included in the study. Following parturition, singletons and twins ewes were categorized into 123 and 109, respectively, weighing 40–60 kg. Two stations—Babylon and Karbala—were randomly selected for these ewes. Concentrates containing 59% barley and 40% bran, along with 1% salt, were fed to animals proportionally to 2.5% of their weight. Three kilograms of alfalfa were also given to each animal, along with 1 kg of straw. All animals had access to fresh water at all times. In the breeding stations, twinning rate, lambing rate, survival rate, and time to lambing were all recorded, as well as age at first lambing.

DNA and polymerase chain reaction

The genetic analysis of the sheep was conducted using a blood sample collected from its jugular vein. Genomic DNA was extracted from a sample using rapid salting-out methods.[23] Using NCBI Primer-BLAST, all 232 *PITX2* genetic sequences were amplified.[24] A polymerase chain reaction (PCR) was performed using a Bioneer premix, and thermal gradients were performed using an Eppendorf thermal gradient apparatus (Germany) to identify the optimal PCR-amplifying conditions (Table 1). The sample was denatured for 4 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, then annealed for 45 seconds and elongated for 30 seconds at 72°C. Then, the PCR products were electrophoresed on agarose gels (2%) (Figure 1), and the gel images were visualized with a Chemidoc Gel Imager (Bio-Rad, Hercules, CA, USA).[25]

Single-strand conformation polymorphism

The genotypes of each PCR product were determined following Imran et al.[6] In equal volume, the denaturing-loading buffer was added to each PCR amplicon (95% formamide, 0.05% xylene cyanol, and 20 mM EDTA, pH 8). Following denaturation for 7 minutes, amplicons were placed on wet ice

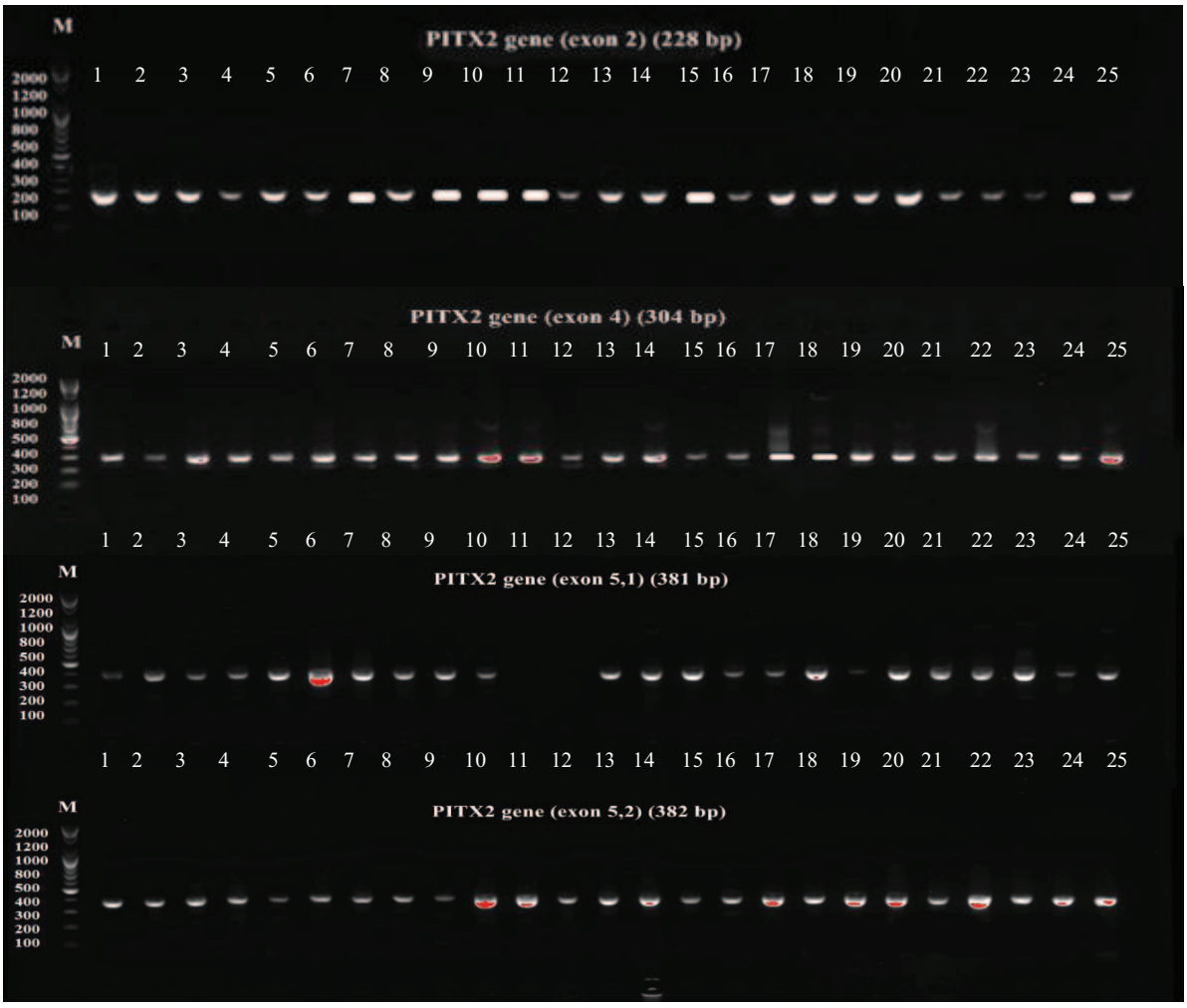

Figure 1. Agarose gel electrophoresis for PCR products from the exon 2 to exon 5 amplification of the *PITX2* gene. Lane M: 100 bp DNA ladder, lanes 1-12 indicate the samples of ewes with a single pregnancy, lanes 13-25 indicate the samples of ewes with twin pregnancies. Electrophoresis conditions: 2% agarose concentration and electrophoresis time: 30 minutes. The buffer used TBE pH 8.3.
PCR indicates polymerase chain reaction; PITX2, paired-like homeodomain transcription factor 2; TBE, Tris-borate-EDTA.

for 10 minutes. Polyacrylamide gels with neutral denaturants were loaded into 0.5 Tris-borate-EDTA (TBE) buffers. For the next step, the gels were electrophoresed for 4 hours at room temperature at 200 mA and 100 V. A protocol described by Byun et al[26] was used to stain the gels.

DNA sequencing

In sequence laboratories (Macrogen, Geum Chen, Korea), all 232 animals were exposed to Sanger-sequencing reactions from both termini after single-strand conformation polymorphism (SSCP) bands were detected on polyacrylamide gels. From the NCBI website (https://www.ncbi.nlm.nih.gov), the *PITX2* gene sequence was retrieved. SnapGene Viewer 4.0.4 (http://www.snapgene.com) was used to visualize the polymorphisms, and BioEdit 7.1 was used to edit polymorphisms within each genotype. The novelty of observed variants was determined using Ensemble genome browser 96 (https://asia.ensembl.org/index.html). On-line prediction software for RNA folding Web Server was used to analyze the effects of genotypes on *PITX2* gene 3' untranslated region (3'UTR) mRNA secondary structure (http://rna.tbi.univie.ac.at/cgi-bin/rnawebsuite/rnafold.cgi).

Statistical analysis

PopGen32, version 1.31,[27] was used in this study to determine the genotype and allele frequencies. Our next step was to determine the Hardy-Weinberg equilibrium (HWE) and then determined the polymorphism information content (PIC) following Botstein et al.[28] IBM SPSS 23.0 (Armonk, NY, USA) was used to analyze the association analysis of *PITX2* genotypes as follows

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

where Y_{ijk} is the phenotypic traits, μ is the mean, G_i is the fixed effect of ith genotypes (i = CC, CT, TT), P_j is the fixed effect of

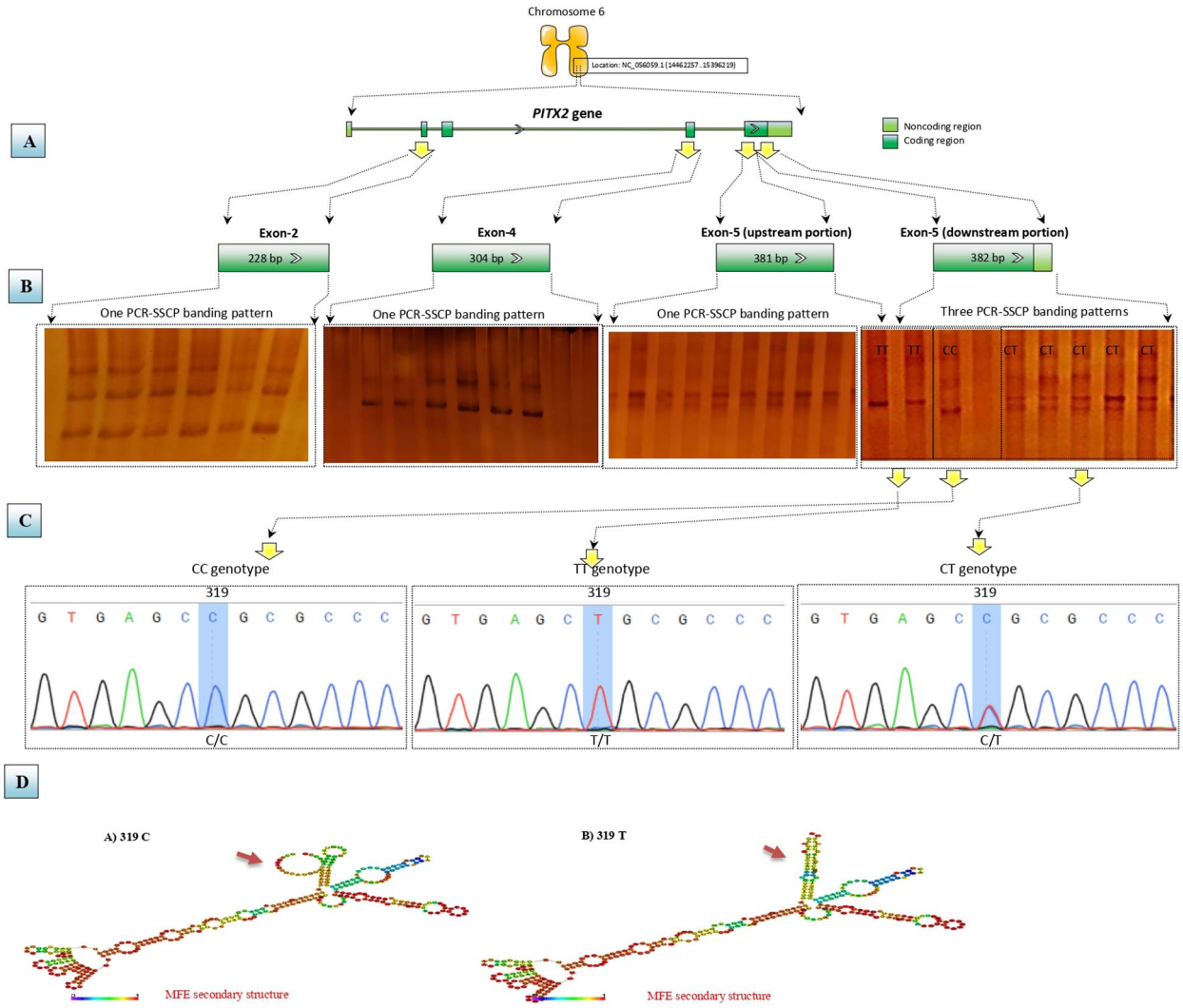

Figure 2 A schematic diagram for the *PITX2* gene-based PCR-SSCP-sequencing strategy within Awassi ewes. (A) PCR design of 4 PCR specific primers pairs for the amplification of 228, 304, 381, and 382 bp in exon 2, exon 4, exon 5 (upstream portion), and exon 5 (downstream portion), respectively. (B) PCR-SSCP genotyping, in which only exon 5 (downstream portion) showed 3 genotypes, homozygous and heterozygous. (C) DNA-sequencing electropherograms of the detected genotypes, in which 1 SNP, 319C > T, was detected in the exon 5 (3'UTR region) in the heterozygous TT genotype. (D) Secondary structure prediction of different genotypes of mRNA in *PITX2* gene 3'-UTR.
PCR indicates polymerase chain reaction; PITX2, paired-like homeodomain transcription factor 2; SNP, single-nucleotide polymorphism; SSCP, single-strand conformation polymorphism.

jth parity ($j = 1, 2, 3$), and e_{ijk} is the random residual error. A significant difference was determined at the (0.05, 0.01) level by the Tukey-Kramer test. Using the chi-square test, 3 reproductive traits were analyzed: lambing rate, survival rate, and type of birth. Paired-like homeodomain transcription factor 2 polymorphisms and litter size were examined using logistic regression. The potential effects of the factor interaction, lambing season, and age were evaluated and discarded when non-significant.

Results

Genotyping, sequencing of PITX2 genes, and genetic diversity

Out of 6 exons, only 3 exons (exons 2, 4, and 5) were, respectively, selected for *PITX2* genotyping in the studied ewes' population. This was because they covered 271 amino acid residues, which make up most paired-like homeodomain 2 amino acid sequences (271 out of 317, or 85.5%). The other exons of the *PITX2* gene were not considered as they covered only 46 amino acid residues of the entire protein (46/317 or 14.5%). The 4 coding regions of the *PITX2* gene, together with their flanking regions, were analyzed by amplification of 4 genetic fragments of 228, 304, 381, and 382 bp, respectively (Figure 2A). For the PCR-SSCP amplicons corresponding to exons 2, 4, and 5 (upstream portion), all electrophoretic migrations were monomorphous, and there was no heterogeneity in the electrophoretic migration as observed for the amplicons of 228, 304, and 381 bp. Polymerase chain reaction-single-strand conformation polymorphism pattern analysis of 382 bp amplicons intended for amplification of exon 5 (downstream portion) revealed 3 distinct patterns (Figure 2B). According to sequencing experiments, there was heterogeneity in exon 5 and 1 polymorphic locus was detected within the amplified exon 5 fragment. The

Table 2. Genetic diversity of the *PITX2* gene in Awassi ewes detected by PCR-SSCP.

OBSERVED GENOTYPES			GENOTYPE FREQUENCIES			ALLELE FREQUENCIES		HO	HE	NE	PIC	χ^2
CC	CT	TT	CC	CT	TT	C	T					
n = 113	n = 40	n = 79	0.42	0.23	0.35	0.57	0.43	0.17	0.49	1.96	0.37	97.96

Abbreviations: χ^2, chi-square; He, expected heterozygosity, Ho, observed heterozygosity, n, number of individuals, Ne, effective allele frequency, PCR, polymerase chain reaction; PIC, polymorphism information content; SSCP, single-strand conformation polymorphism.
All chi-square tests have 2 degrees of freedom and within the significance level $P \leqslant .05$.

Table 3. The association between *PITX2* genetic polymorphism and reproductive performance in Awassi ewes.

GENOTYPES	BIRTH TYPE (%)		LAMBING RATE (%)	SURVIVAL RATE (%)	DAYS TO LAMBING (LSM ± SE)	AGE AT FIRST LAMBING (LSM ± SE)
	SINGLETON	TWIN				
CC	34 (30.08%)	79 (69.91%)	96	189 (98.43%)	$161^a \pm 8.62$	519.41 ± 26.32
CT	23 (57.50%)	17 (42.50%)	88	55 (96.49%)	$170^b \pm 9.31$	523.11 ± 28.41
TT	58 (73.41%)	21 (26.58%)	82	96 (96.00%)	$184^c \pm 11.48$	534.32 ± 29.12
P value	**.001**	**.01**	**.03**	.31	**.02**	.23

Abbreviations: LSM ± SE, least square means ± standard error; PITX2, paired-like homeodomain transcription factor 2.
[a,b,c]Significant differences in mean values represent by differences in the same column within each classification, the *P* value with statistical significance are indicated in bold numbers.

variants belonging to CC, CT, and TT genotypes were predicted from the 319C > T nucleic acid substitution detected in the PCR amplicons, which corresponds to homozygous C/C, T/T, and heterozygous C/T patterns (Figure 2C). The different genotypes (CC, TT, and CT) were classified based on the SSCP pattern and confirmed based on the Sanger sequencing. Samples that did not appear for the first time in the gel were reposted to get the best picture. On-line prediction software RNA fold Web Server was used to determine the effects of genotypes on *PITX2* gene 3'UTR mRNA secondary structure. The 319C > T mutation altered the secondary structure of *PITX2* 3'UTR in these genotypes (Figure 2D).

Based on genetic diversity, there were 113 genotypes of the CC genotype, with a total frequency of 0.42. Following this were the TT and CT genotypes, with frequencies of 0.35 and 0.23, respectively. Expected heterozygosity (He) values were higher than observed heterozygosity (Ho) values at the 319C > T SNP locus, indicating lower genetic variation (Table 2). Based on the classification of PIC (low, median, and high polymorphism if PIC value < 0.25, 0.25 < PIC value < 0.5, and PIC value > 0.5, respectively), this study showed a moderate level of polymorphism at the 319C > T SNP locus. The chi-square analysis showed significant deviance from the HWE for the 319C > T SNP locus of the *PITX2* gene ($P \leqslant .05$).

Association analysis of PITX2 gene with reproductive performance

Based on the 319C > T SNP, an association analysis found no significant difference ($P \geqslant .01$) between CC and CT/TT genotypes in survival rate and age of first lambing. The CC genotype at the same 319C > T locus was associated significantly ($P \leqslant .01$) with more litter sizes, twinning rates, lambing rates, and shorter lambing days compared with the CT and TT genotypes (Table 3). An analysis of logistic regression provided further insights into the association between 319C > T and litter size in Table 4. Ewes with CC genotypes had 1.70 lambs per animal compared with CT and TT genotypes. As a result, the SNP 319C > T negatively impacted these traits.

Discussion

Several studies have reported genetic variations in the *PITX2* gene in livestock. In 2 famous Chinese dairy goat breeds, 4 single-nucleotide polymorphisms (SNPs) of the caprine *PITX2* gene have been found: g.18117TNC, g.18161CNG, g.18322CNA, and g.18353TNC. These SNPs are associated significantly with dairy goat milk traits.[12] Furthermore, genetic variant g.18117T > C, g.18353T > C, and g.18161C > G within the caprine *PITX2* genes was correlated significantly with growth traits in Guanzhong dairy goats, according to Zhang et al.[29] In chickens, the novel SNPs g.12713A > G of the *PITX2* gene is associated significantly with carcass characteristics in Wuliang Mountain Black-bone hens.[20] Recently, 4 SNPs (g.9830C > T, g.13335G > A, g.10073 C > T, g.13726A > G) of the *PITX2* gene showed significant association with chicken meat quality.[13] However, the literature about *PITX2* variation in Awassi sheep is rare.

In this study, the 319C > T SNP occurred in a non-coding region and significantly affected economic traits. A possible explanation for this situation is that the conserved non-coding region of DNA has previously been found to play a vital role in biological reactions.[30] In the case of synonymous mutations, the

Table 4. Logistic regression analysis of *PITX2* genotype and litter size in Awassi ewes.

GENOTYPE	LITTER SIZE (LSM ± SE)	LOGISTIC REGRESSION ANALYSIS		
		β	ODDS RATIO (95%CI)	*P* VALUE
CC	1.70[a] ± 0.15	1.00	Reference	**.001**
CT	1.43[b] ± 0.10	–1.03	2.80 (1.49-5.62)	
TT	1.27[c] ± 0.09	–1.32	3.85 (1.38-6.18)	**.001**

Abbreviations: β, regression coefficients; CI, confidence interval; LSM ± SE, least square means ± standard error; PITX2, paired-like homeodomain transcription factor 2.
[a,b,c]Significant differences in mean values represent by differences in the same column within each classification, the *P* value with statistical significance are indicated in bold numbers.

coded message remains unchanged. However, protein production may be affected by impaired RNA splicing. Furthermore, it can lower the translation of gene products and influence regulatory sequences.[31] A further study by Manning and Cooper[32] revealed that non-coding variants found in RNA synthesis regions are linked to post-translational processing and translation initiation. Various functions of the genome may be affected by changes to RNA secondary structures, including protein folding, accessibility of RNA to functional sites, alternative splicing, and proteins that recognize RNA binding sites.[33] A recent study indicates that the 3'UTR region of genes can influence mRNA location and protein abundance through their effects on mRNA stability and translation.[34] Ribeiro et al[35] revealed that genetic information stored in gene 3'UTR can affect protein expression through protein-protein interactions.

Statistically, individuals belonging to the CC genotype had higher litter sizes, twinning rates, lambing rates, and fewer days to lambing than those belonging to the CT and TT genotypes. As a result of these findings, the 319C > T mutation negatively affects the reproductive performance of Awassi ewes. The PITX1 and PITX2 proteins affect several fertility genes, such as *GH, PRL*, *LH*β, *FSH*β, and *GnRH* receptors by interactions with cell-specific factors, including NeuroD1/Pan1, Steroidogenic Factor-1 (SF-1), and Pit1.[14] PITX2 may regulate the function of the ovary by interacting with its cofactor Pit1. Paired-like homeodomain transcription factor 2 may also regulate unidentified genes and could play a role in steroidogenesis, folliculogenesis, and ovarian development.[36] PITX2 contributes significantly to reproductive traits because, as a member of the POU1F1-PROP1 pathway, *and regulates the expression of POU1F1*, *LHX3*, *PROP1*, *GH*, and *PRL* genes.[17] In addition, PITX2 regulates Wnt/β-catenin pathways, which are involved in embryo implantation, linking this to litter size.[9,15] However, a few reports in the literature describe the impact of non-coding region SNPs, specifically the 3'UTR, on phenotypic traits. In goat *PITX2* genes, a novel 22-bp indel mutation is associated with litter size and growth characteristics.[9] According to Jia et al,[37] an occurrence of synonymous mutations of the bone morphogenetic protein receptor type 1B (*BMPR-1B*) gene in Mongolian, Dorset, and Small Tail Han sheep can cause the premature termination of mRNA, which affects sheep follicular oocyte expression and granulosa cells. A novel variant, g.46544883A > G within the 3' UTR of *GDF9*, has been shown to affect litter size in Mongolian ewes.[38] Jia et al[39] found that the A1354G SNP of the 3'UTR region of the *BMPR-1B* gene is correlated with litter size. Statistical analysis indicated that the 13-bp indel substitution in the 3' UTR region of the A-kinase anchoring protein 12 (*AKAP12*) gene is correlated significantly with litter size in Shaanbei white cashmere goats and those carriers with DD genotypes produced smaller litters compared with carriers of ID and II genotypes.[40] Based on these data, little is known about how this gene polymorphism affects sheep reproductive performance. The Awassi sheep is a prevalent breed throughout most Middle Eastern countries.[41] Although this breed is known for its ability to cope with unfavorable conditions,[42] its reproduction is lower than other breeds in the region, such as Karakuls and Assafs.[3] Most Middle Eastern breeders are concerned about the low reproductive capacity of this breed, which drives efforts to improve its reproductive performance. Based on this, the *PITX2* gene appears to have the potential for influencing reproductive performance in Awassi sheep. The polymorphism of the *PITX2* gene is strongly suggested as a new critical candidate in marker-assisted selection for future improvement of the litter size of the Awassi ewe's population.

Conclusions

A novel SNP, 319C > T, was found in the heterozygous CT genotype of the *PITX2* gene (3'UTR). Ewes with the CC genotype showed significant associations with higher litter sizes, twinning rate, lambing rates, and shorter days to lambing than ewes with the CT and TT genotypes. Ewes with CC genotypes showed better measurements of reproductive traits than those with TT genotypes. Based on the results of this study, ewes carrying the 319C > T SNP have a lower litter size and are less prolific than ewes without the SNP.

Acknowledgements

The authors thank Babylon and Karbala sheep stations for providing the ewes for their study.

Author Contributions

All authors contributed equally.

REFERENCES

1. Maquivar MG, Smith SM, Busboom JR. Reproductive management of rams and ram lambs during the pre-breeding season in US sheep farms. *Animals*. 2021;11:2503. doi:10.3390/ani11092503
2. AL-Thuwaini TM. Adiponectin and its physiological function in ruminant livestock. *Rev Agric Sci*. 2022;10:115-122. doi:10.7831/ras.10.0_115
3. Ajafar MH, Kadhim AH, AL-Thuwaini TM. The reproductive traits of sheep and their influencing factors. *Rev Agric Sci*. 2022;10:82-89. doi:10.7831/ras.10.0_82
4. Gholizadeh M, Esmaeili-Fard SM. Meta-analysis of genome-wide association studies for litter size in sheep. *Theriogenology*. 2022;180:103-112. doi:10.1016/j.theriogenology.2021.12.025
5. Ajafar MH, Al-Thuwaini TM, Dakhel HH. Association of OLR1 gene polymorphism with live body weight and body morphometric traits in Awassi ewes. *Mol Biol Rep*. 2022;49:4149-4153. doi:10.1007/s11033-022-07481-3
6. Imran FS, Al-Thuwaini TM, Al-Shuhaib MBS, Lepretre F. A novel missense single nucleotide polymorphism in the GREM1 gene is highly associated with higher reproductive traits in Awassi sheep. *Biochem Genet*. 2021;59:422-436. doi:10.1007/s10528-020
7. Al-Thuwaini TM. Novel single nucleotide polymorphism in the prolactin gene of Awassi ewes and its role in the reproductive traits. *Iraqi J Vet Sci*. 2021;35:429-435. doi:10.33899/ijvs.2020.126973.1423
8. Mohammed MM, Al-Thuwaini TM, Al-Shuhaib MBS A novel p. K116Q SNP in the OLR1 gene and its relation to fecundity in Awassi ewes. *Theriogenology*. 2022;184:185-190. doi:10.1016/j.theriogenology.2022.03.014
9. Yan H, Jiang E, Zhu H, Hu L, Liu J, Qu L. The novel 22 bp insertion mutation in a promoter region of the PITX2 gene is associated with litter size and growth traits in goats. *Arch Anim Breed*. 2018;61:329-336. doi:10.5194/aab-61
10. Zhang S, Xu H, Kang Z, et al. Bovine pituitary homeobox 2 (PITX2): mRNA expression profiles of different alternatively spliced variants and association analyses with growth traits. *Gene*. 2018;669:1-7. doi:10.1016/j.gene.2018.05.083
11. Hernandez-Torres F, Rodríguez-Outeiriño L, Franco D, Aranega AE. Pitx2 in embryonic and adult myogenesis. *Front Cell Dev Biol*. 2017;5:46. doi:10.3389/fcell.2017.00046
12. Zhao H, Wu X, Cai H, et al. Genetic variants and effects on milk traits of the caprine paired-like homeodomain transcription factor 2 (PITX2) gene in dairy goats. *Gene*. 2013;532:203-210. doi:10.1016/j.gene.2013.09.062
13. Cao H, Zhou W, Tan Y, et al. Chronological expression of PITX2 and SIX1 genes and the association between their polymorphisms and chicken meat quality traits. *Animals*. 2021;11:445. doi:10.3390/ani11020445
14. Guioli S, Lovell-Badge R. PITX2 controls asymmetric gonadal development in both sexes of the chick and can rescue the degeneration of the right ovary. *Development*. 2007;134:4199-4208. doi:10.1242/dev.010249
15. Majidinia M, Aghazadeh J, Jahanban-Esfahlani R, Yousefi B. The roles of Wnt/β-catenin pathway in tissue development and regenerative medicine. *J Cell Physiol*. 2018;233:5598-5612. doi:10.1002/jcp.26265
16. Kioussi C, Briata P, Baek SH, et al. Identification of a Wnt/Dvl/β-catenin→Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell*. 2002;111:673-685.
17. Zhao H, He S, Zhu Y, et al. A novel 29 bp insertion/deletion (indel) variant of the LHX3 gene and its influence on growth traits in four sheep breeds of various fecundity. *Arch Anim Breed*. 2017;60:79-85. doi:10.5194/aab-60
18. Torlopp A, Khan MA, Oliveira NM, et al. The transcription factor Pitx2 positions the embryonic axis and regulates twinning. *Elife*. 2014;3:e03743. doi:10.7554/eLife.03743
19. Quan Q, Zhu L, Zheng Q, et al. Comparison of the pituitary gland transcriptome in pregnant and non-pregnant goats (*Capra hircus*). *Czech J Anim Sci*. 2019;64:420-430. doi:10.17221/141/2019-CJAS
20. Cao H, Dong X, Mao H, Xu N, Yin Z. Expression analysis of the PITX2 gene and associations between its polymorphisms and body size and carcass traits in chickens. *Animals*. 2019;9:1001. doi:10.3390/ani9121001
21. Priston M, Kozlowski K, Gill D, et al. Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld–Rieger syndrome. *Hum Mol Genet*. 2001;10:1631-1638. doi:10.1093/hmg/10.16.1631
22. Jiang Y, Tang S, Wang C, et al. A genome-wide association study of growth and fatness traits in two pig populations with different genetic backgrounds. *J Anim Sci*. 2018;96:806-816. doi:10.1093/jas/skx038
23. Al-Shuhaib Mohammed Baqur SA. A universal, rapid, and inexpensive method for genomic DNA isolation from the whole blood of mammals and birds. *J Genet*. 2017;96:171-176. doi:10.1007/s12041-017
24. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform*. 2012;13:1-11. doi:10.1186/1471-2105
25. Al-Thuwaini T. Association between polymorphism in BMP15 and GDF9 genes and impairing female fecundity in diabetes type 2. *Middle East Fertil Soc J*. 2020;25:1-10. doi:10.1186/s43043-020
26. Byun SO, Fang Q, Zhou H, Hickford JGH. An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Anal Biochem*. 2009;385:174-175. doi:10.1016/j.ab.2008.10.024
27. Yeh FC, Yang RC, Boyle T. *Microsoft Window-Based Freeware for Population Genetic Analysis (POPGENE), Ver. 1.31*. University of Alberta; 1999.
28. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet*. 1980;32:314-331.
29. Zhang S, Zhao H, Lei C, et al. Effects of genetic variations within goat PITX2 gene on growth traits and mRNA expression. *Anim Biotechnol*. 2020;31:107-114. doi:10.1080/10495398.2018.1551229
30. Alexander RP, Fang G, Rozowsky J, Snyder M, Gerstein MB. Annotating non-coding regions of the genome. *Nat Rev Genet*. 2010;11:559-571. doi:10.1038/nrg2814
31. Li D, McIntosh CS, Mastaglia FL, Wilton SD, Aung-Htut MT. Neurodegenerative diseases: a hotbed for splicing defects and the potential therapies. *Transl Neurodegener*. 2021;10:1-18. doi:10.1186/s40035-021
32. Manning KS, Cooper TA. The roles of RNA processing in translating genotype to phenotype. *Nat Rev Mol Cell Biol*. 2017;18:102-114. doi:10.1038/nrm.2016.139
33. Li MJ, Yan B, Sham PC, Wang J. Exploring the function of genetic variants in the non-coding genomic regions: approaches for identifying human regulatory variants affecting gene expression. *Brief Bioinform*. 2015;16:393-412. doi:10.1093/bib/bbu018
34. Zhu S, Zhang L, Ma X, Zhang X. Analysis and differences of sheep BMP-15 gene expression in ewes with different lambing traits. *Genomics Appl Biol*. 2018;37:1853-1858. doi:10.13417/j.gab.037.001853
35. Ribeiro DM, Prod'homme A, Teixeira A, Zanzoni A, Brun C. The role of 3′ UTR-protein complexes in the regulation of protein multifunctionality and subcellular localization. *Nucleic Acids Res*. 2020;48:6491-6502. doi:10.1093/nar/gkaa462
36. Ghosh P, Saha SK, Nandi SS, Bhattacharya S, Roy SS. Involvement of Pitx2, a homeodomain transcription factor, in hypothyroidism associated reproductive disorders. *Cell Physiol Biochem*. 2007;20:887-898. doi:10.1159/000110449
37. Jia J, Chen Q, Gui L, et al. Association of polymorphisms in bone morphogenetic protein receptor-1B gene exon-9 with litter size in Dorset, Mongolian, and small Tail Han ewes. *Asian-Australas J Anim Sci*. 2019;32:949-955. doi:10.5713/ajas.18.0541
38. Tong B, Wang J, Cheng Z, et al. Novel variants in GDF9 gene affect promoter activity and litter size in Mongolia sheep. *Genes*. 2020;11:375. doi:10.3390/genes11040375
39. Jia J, Xie D, Zhang Y, et al. Association of BMPR-1B Gene 3'-UTR region polymorphism with litter size in Tibetan sheep. *Kafkas Univ Vet Fak Derg*. 2021;27:285-289. doi:10.9775/kvfd.2020.25064
40. Kang Z, Bai Y, Lan X, Zhao H. Goat AKAP12: indel mutation detection, association analysis with litter size and alternative splicing variant expression. *Front Genet*. 2021;12:648256. doi:10.3389/fgene.2021.648256
41. Al-Thuwaini TM, Kareem ZA. Novel missense variant L46Q of fatty acid synthase gene and fatty acids content in Awassi sheep. *Acta Sci-Anim Sci*. 2022;44:e56273-e56273. doi:10.4025/actascianimsci.v44i1.56273
42. Al-Thuwaini TM. The relationship of hematological parameters with adaptation and reproduction in sheep; a review study. *Iraqi J Vet Sci*. 2021;35:575-580. doi:10.33899/ijvs.2020.127253.1490