A Missense p.Q>R234 Mutation in the Osteopontin Gene Is Associated With the Prolificacy of Iraqi Awassi Ewes

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ABSTRACT: One of the most valuable traits in production and breeding is a sheep's prolificacy which is influenced by several genes, one of which is the osteopontin (*OPN*) gene. Thus, this study aimed to determine the effect of genetic variation within the *OPN* gene on Awassi ewe prolificacy. Genomic DNA was extracted from 123 single-progeny ewes and 109 twin ewes. Polymerase chain reaction (PCR) was used to amplify 4 sequence fragments (289, 275, 338, and 372 bp), representing exons 4, 5, 6, and 7 of the *OPN* gene. A 372 bp amplicon was identified with 3 different genotypes: TT, TC, and CC. Sequence analysis revealed a novel mutation in TC genotypes p.Q>R234. Statistical analysis revealed that the single nucleotide polymorphism (SNP) p.Q>R234 was associated with prolificacy. Ewes carrying the p.Q>R234 SNP had significantly ($P \leq .01$) lower litter sizes, twinning rates, and lambing rates, and more days to lambing than those with the TC and TT genotypes. The p.Q>R234 SNP was confirmed to be responsible for lower litter size through logistic regression analysis. From these results, we can conclude that the missense variant p.Q>R234 adversely affects the traits of interest and shows that the p.Q>R234 SNP negatively influences the prolificacy of Awassi sheep. Based on this study, it is evident that ewes in this population carrying the p.Q>R234 SNP have a lower litter size and are less prolific.

KEYWORDS: In silico, litter size, osteopontin, polymorphism, sheep

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Introduction

Prolificacy, ovulation rate, litter size, and age at first lambing are all reproductive traits that can influence the economy of sheep production. The first 2 characteristics have the highest economic value in the Middle East.[1,2] Prolificacy is one of the most economically important traits in production and sheep breeding that is influenced by both genetic and environmental factors. The genetic mechanisms of prolificacy in domestic sheep (*Ovis aries*) remain poorly understood despite their importance.[3] Numerous genes have been associated with prolificacy and other reproductive traits in Awassi sheep, including the *GREM1* gene,[4] the prolactin gene,[5] the *OLR1* gene,[6] and many other candidate genes,[7] one of which is osteopontin (*OPN*), which is related to reproduction and fertility.[8] The *OPN* gene resides on sheep chromosome 6 and is composed of 8 exons.[9] It also resides on the autosome of *Bos taurus* (BTA) 6 with 6 exons.[10] This gene-encoded OPN protein, also known as secreted phosphoprotein 1 (SPP1), is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of extracellular matrix proteins/cytokines and has ~300 amino acids and ~30 carbohydrate residues attached.[11] Osteopontin is expressed initially in osteoblasts, as well as in ovarian follicles and ovaries, and in the myometrium and endometrium in the reproductive tissues.[12] Uterine epitheliums (glands and luminal) express this protein, which interacts with the embryo using integrins to promote adhesion and communication between the uterus and the developing embryos.[13] In particular, OPN is persistently expressed on the maternal-fetal interface, which suggests that OPN is also crucial for maintaining a microenvironment that promotes embryonic and placental development.[8,14] Placental traits and prolificacy in goats are influenced genetically by *OPN* mutations. This may be due to the fact that OPN appears to play a key role in conception, implantation, and pregnancy maintenance.[14] Moreover, many variations have been identified in the *OPN* (*SPP1*) gene with production traits in cattle,[15] and reproductive traits and litter size in pigs.[13,16,17] Based on the above literature review, very few studies have been conducted on genetic polymorphisms in the *OPN* gene in relation to their association with cattle prolificacy, and to the best of our knowledge, no studies have been conducted regarding their association with sheep prolificacy.

The Awassi breed of sheep is the most widespread in the Middle East.[18] Although this breed is known for its ability to adapt to harsh conditions,[19] it has been reported as being less prolific than other breeds in the region.[5] Middle Eastern breeders' main concern is the low reproductive capacity of the Awassi sheep, which drives their efforts to improve this breed's reproductive capacity. Based on this, breeding improvements in Awassi sheep may be possible using the *OPN* gene. To date, only a few studies have investigated the relationship between genetic polymorphisms in the *OPN* gene and prolificacy in sheep, and no studies have investigated their correlation with prolificacy in Awassi sheep. Therefore, this study was conducted to determine whether polymorphisms in the *OPN* gene code region contribute to the prolificacy of Awassi sheep. Using a variety of state-of-the-art in silico tools, this study evaluated the genetic diversity and polymorphism of the *OPN* gene in the study population and explored the deleterious effects of p.Q>R234 single nucleotide

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

PRIMER CODE	LOCUS	SEQUENCE (5' -3')	BINDING COORDINATE IN THE GENOME		AMPLICON LENGTH, BP	ANNEALING TEMPERATURE (°C)
			START	STOP		
OPN, exo4-F	Exon 4	CTCAAATGGCCTGCTTGGTG	37372609	37372628	289	59.8
OPN, exo4-R		TGCTTATCTGTCAAGATTGGGG	37372897	37372876		
OPN, exo5-F	Exon 5	TGCTAACGGAGTTTTGCCAGA	37371999	37372019	275	58.6
OPN, exo5-R		TGCCCGCTTAATCCTTCCAC	37372273	37372254		
OPN, exo6-F	Exon 6	ACCTCAACGTTAGATCGGCG	37371019	37371038	338	61.0
OPN, exo6-R		CCTGACACCCATTTTTCTGGC	37371356	37371336		
OPN, exo7-F	Exon 7	TCAGTTGACCTCAGAAGAGACAC	37370015	37370037	372	58.6
OPN, exo7-R		GCTTCCCTCCCTAGCTGTTC	37370386	37370367		

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.
Abbreviation: *OPN*, osteopontin.

polymorphism (SNP) on structure, function, and stability of the OPN protein. Next, the *OPN* genotypes of Awassi ewes were analyzed concerning litter size, lambing rate, number of days to lambing, and age of first lambing. The association between litter size and *OPN* variants was also determined.

Materials and Methods

Animal

This research was conducted at Al-Qasim Green University from July 2021 to April 2022 following international guidelines for animal care and use (Agri, No. 015,7,20). The study included 232 sexually mature ewes, multiparous between the ages of 3 and 4 years. The sample consisted of 123 singletons and 109 twins collected randomly from 2 sheep stations—Babylon and Karbala—that were classified according to their reproductive history with weights ranging from 40 to 60 kg. In high-prolific ewes, more twins were born than singles. Low-prolific ewes gave birth to more singles than twins. All animals were fed individually and kept in similar nutrition conditions. In proportion to 2.5% of their weight, animals were fed daily concentrates of 59% barley and 40% bran, along with 1% salt. Each animal also received 1 kilogram of straw along with 3 kilograms of green alfalfa. Fresh water was available at all times for all animals. Numerous prolific traits were recorded at the breeding stations, including twinning rate (is the propensity to have more twin litters per 100 ewes at the same average lambing percentage), lambing rate, number of days to lambing (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).

Genomic DNA extraction and PCR amplification

A jugular vein blood sample was collected to analyze the sheep's genetic data. Genomic DNA was extracted using the rapid salting-out method.[20] Amplification of the *OPN* genetic sequence for all 232 animals was conducted using NCBI Primer-BLAST.[21] A PCR experiment was performed using a Bioneer premix (50 µM, 10 mM, 30 mM, 1.5 mM for dNTPs, Tris-HCl, KCl, MgCl2, and 1 U Top DNA polymerase). The best PCR amplification conditions were identified using the thermal gradient device (Eppendorf, Germany) (Table 1). Denaturation was carried out for 4 minutes at 94 °C, followed by 30 cycles of 30 seconds at 94 °C, 45 seconds at annealing, and 30 seconds at 72 °C for elongation. The PCR products were electrophoresed on agarose gels (2%), and gel images were taken with a Chemidoc Gel Imager (Bio-Rad, USA).[22]

Single-strand conformation polymorphism

All PCR products were genotyped according to Imran et al.[4] For each PCR amplicon, equal volumes of the denaturing-loading buffer were added (95% formamide, 0.05% xylene cyanol, and 20 mM EDTA, pH 8). Denaturing the PCR amplicons for 7 minutes was followed by transferring them to wet ice and storing them for 10 minutes. In polyacrylamide gels with a neutral denaturant, samples were loaded in a 0.5 Tris/Borate/EDTA (TBE) buffer. In the following steps, the gels were exposed to electrophoresis at 200 mA and 100 V for 4 hours at room temperature. The gels were stained according to the rapid staining protocol developed by Byun et al.[23]

DNA sequencing and in silico analysis

On detecting single-strand conformation polymorphism (SSCP) bands on polyacrylamide gels, all 232 animals were subjected to downstream reactions at sequence laboratories (Macrogen, Geum Chen, Korea). A sequence of the *OPN* gene was retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov). The DNA polymorphisms within each genotype were edited using BioEdit 7.1 version (DNASTAR, Madison)

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

OBSERVED GENOTYPES			GENOTYPE FREQUENCIES			ALLELE FREQUENCIES		*HO*	*HE*	*NE*	PIC	χ^2
TT	TC	CC	TT	TC	CC	T	C					
n = 97	*n* = 53	*n* = 82	0.42	0.23	0.35	0.53	0.47	0.22	0.49	1.99	0.37	68.49

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

and visualized using SnapGene Viewer 4.0.4 (http://www.snapgene.com). Ensemble genome browser 96 was used to determine the novelty of the observed variants (https://asia.ensembl.org/index.html). The Expasy software was used to detect amino acid reading frames.[24] The next step was comparing amino acid sequences with those in the OPN database using UniProtKB (http://www.uniprot.org/align/). The structure and function of mutant proteins were predicted using a variety of computational tools, including SIFT,[25] PolyPhen-2,[26] I-Mutant2,[27] Mupro,[28] CUPsat,[29] and INPS.[30]

Statistical analysis

This study determined the genotype and allele frequencies using PopGen32, version 1.31.[31] Afterward, the Hardy-Weinberg equilibrium was determined, and we followed Botstein et al[32] to determine the polymorphism information content (PIC). Association analysis of *OPN* genotypes was performed using IBM SPSS 23.0 (NY, USA) as follows

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

where *Yijk* = phenotypic traits, μ = mean, *Gi* = fixed effect of *i*th genotypes (*i* = TT, TC, CC), *Pj* = fixed effect of *j*th parity (*j* = 1, 2, 3), and *eijk* = random residual error. The Tukey-Kramer test was used to determine whether there was a significant difference between the means at the .05 level. Lambing rate (number of lambs per ewe exposed to a ram) and birth type were 2 reproductive traits analyzed with the χ^2 test. Logistic regression was used to examine the association between *OPN* polymorphisms and litter size. Preliminary results indicate that interactions, lambing season, station, and age were not affected by the model results and were excluded.

Results

Genetic diversity, genotyping of OPN gene, and sequencing analysis

Genetic diversity of the *OPN* gene showed that the most common genotype was TT (n = 97), with a total frequency of 0.42. Second was the CC genotype (n = 82), with a frequency of 0.35, followed by the TC genotype (n = 53), with a frequency of 0.23. Genetic variation was found to be high at the c.137T>C SNP locus, which was reflected by higher He values than Ho values (Table 2). The present study indicated a moderate level of PIC at the c.137T>C SNP locus based on the classification of PIC (low polymorphism if PIC value < 0.25; median polymorphism if 0.25 < PIC value < 0.5, and high polymorphism if PIC value > 0.5). The χ^2 test showed that the polymorphism of the *OPN* gene at the c.137T>C SNP locus deviated significantly from the HWE ($P \leqslant .05$).

Out of 8 exons, only 4 exons (exons 4, 5, 6, and 7) were respectively selected for *OPN* genotyping in the studied ewes' population. This was because they covered 240 amino acid residues, which make up most OPN amino acid sequences (240 of 278, or 86.3%). The other exons of the *OPN* gene were not considered as they covered only 38 amino acid residues of the entire protein (38 of 278, or 13.6%). An amplification of 4 genetic fragments of 289, 275, 338, and 372 bp was performed to analyze the 4 coding regions of the *OPN* gene alongside their flanking regions (Figure 1A). All PCR-SSCP amplicons corresponding to exons 4, 5, and 6 showed monomorphous electrophoretic migrations and no heterogeneity was observed for 289, 275, and 338 bp amplicons. The 372 bp amplicons intended to amplify exon 7 displayed 3 distinct PCR-SSCP band patterns (Figure 1B). Sequencing experiments showed that the c.137T>C SNP was present only in one of the SSCP variants, indicating the presence of heterogeneity in exon 7. Based on the detected c.137T>C nucleic acid substitution, the identified variants were assigned TT, TC, and CC genotypes, corresponding to the homozygous T/T and C/C, and the heterozygous T/C patterns observed at 137 position in the coding sequencing of the exon 7 amplicons, respectively (Figure 1C). Expasy software identified a missense consequence of this SNP, which resulted in glutamine-to-arginine substitution at the 234th position of the mature OPN protein (p.Q>R234) (Figure 1D). Seven in silico tools (SIFT, PolyPhen-2, I-Mutant2, Mupro, SUSPect, CUPSAT, and INPS) were used to investigate the consequences of the detected p.Q234R change on OPN structure, stability, and function. A high amount of deleterious effects were predicted by all computational analyses (Table 3, Figure 1D). According to these tools, this nsSNP might negatively influence the scheduled biological activities of those with CC genotypes as compared with those with TT genotypes.

Association analysis of OPN gene with prolificacy

Association analysis determined no significant ($P \geqslant .01$) differences in the age of first lambing for genotyped TT and TC/CC

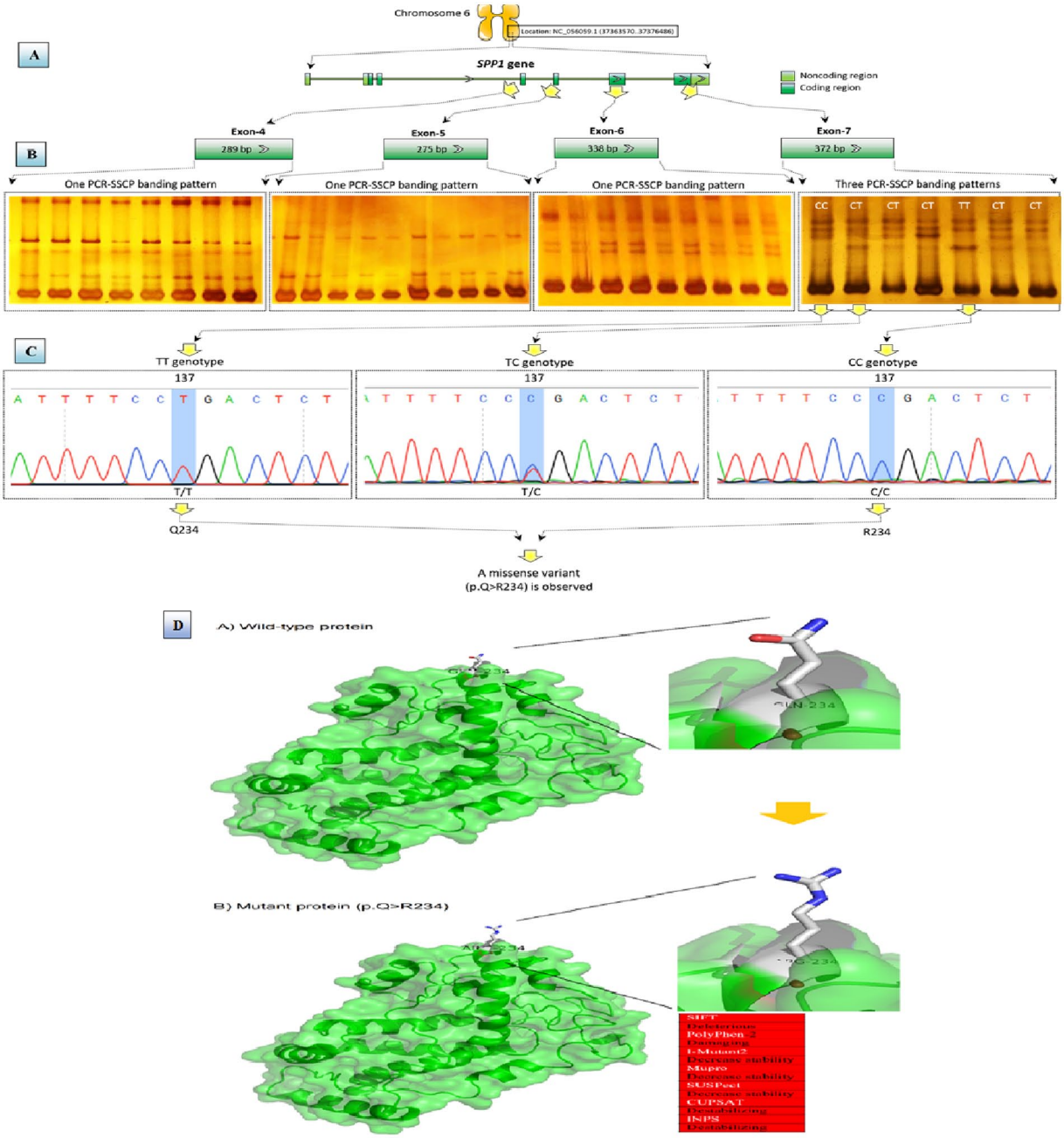

Figure 1. A schematic diagram for the *OPN* gene-based PCR-SSCP-sequencing strategy within Awassi ewes. (A) PCR designs of 4 PCR-specific primers pair, intended to amplify 289, 275, 338, and 372 bp in exons 4, 5, 6, and 7. (B) Genotyping by PCR-SSCP, in which only exon 7 showed homozygous and heterozygous genotypes. (C) The electropherogram of DNA sequencing data, showing a single SNP, c.137T>C, in exon 7 in the heterozygous TC genotype. (D) Implementation of 7 bioinformatics techniques to study the deleterious effects of the p.Q>R234 SNP on the OPN protein. OPN indicates osteopontin; PIC, polymorphism information content; SNP, single nucleotide polymorphism; SSCP, single-strand conformation polymorphism.

ewes based on the p.Q>R234 SNP. At the same p.Q>R234 locus, the TT genotype was associated significantly ($P \leq .01$) with larger litter size, a higher twinning rate, a higher lambing rate, and fewer days to lambing as compared with TC and CC genotypes (Table 4). Further insights into the association between p.Q>R234 and litter size were obtained using logistic regression analysis in Table 5. A TT genotype ewe had 1.71 lambs per animal compared with a TC and CC genotype ewe. Consequently, the missense SNP p.Q>R234 negatively affected these traits.

Discussion

Several studies have investigated *OPN* gene variants and their association with productive traits in livestock. Leonard et al[33] determined 3 genotypes—CC, CT, and TT—of the *OPN* in dairy cattle based on the polymorphism in the intronic regions

Table 3. The in silico prediction of p.Q>R234 on ovine OPN protein, in terms of structure and function.

IN SILICO TOOLS	SCORE	PREDICTION
SIFT	0.00	Deleterious
PolyPhen-2	0.81	Damaging
Mupro	ΔG (−0.648 kcal/mol)	Decrease stability
SUSPect	Grade 6	Decrease stability
CUPSAT	ΔG (−0.94 kcal/mol)	Destabilizing
I-Mutant2	DDG (−0.05 kcal/mol)	Decrease stability
INPS	ΔG (−0.355 kcal/mol)	Destabilizing

Abbreviation: OPN, osteopontin.

Table 4. The association between *OPN* genetic polymorphism at locus p.Q>R234 and reproductive performance in Awassi ewes.

GENOTYPES	BIRTH TYPE (%)		LAMBING RATE (%)	DAYS TO LAMBING (LSM ± SE)	AGE AT FIRST LAMBING (LSM ± SE)
	SINGLETON	TWIN			
TT	28 (28.87%)	69 (71.13%)	94	159 ± 11.9[a]	521.03 ± 28.14[a]
TC	34 (64.15%)	19 (35.84%)	86	170 ± 12.3[ab]	528.14 ± 32.09[a]
CC	61 (74.39%)	21 (25.60%)	82	178 ± 14.6[b]	533.21 ± 24.25[a]
P value	.001***	.001***	.01**	.02**	.41

Abbreviations: LSM ± SE, least square means ± Standard error; OPN, osteopontin.
[a, b] Significant differences in means represented by differences in the same column within each classification.
*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$.

Table 5. Logistic regression analysis of p.Q>R234 with litter size in Awassi ewes.

GENOTYPE	LITTER SIZE (LSM ± SE)	LOGISTIC REGRESSION ANALYSIS		
		β	ODDS RATIO (95% CI)	*P* VALUE
TT	1.71 ± 0.10[a]	1.00	Reference	.001***
TC	1.35 ± 0.03[ab]	−0.48	1.61 (1.11-4.63)	
CC	1.26 ± 0.12[b]	−1.06	2.88 (1.40-6.51)	.001***

Abbreviations: β, regression coefficient; LSM ± SE, least square means ± Standard error; CI: confidence interval.
[a, b] Significant differences in means represented by differences in the same column within each classification.
*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$.

of the gene. Meanwhile, using BseNI/PCR-RFLP, Oztabak et al[10] identified 3 genotypes of *OPN* in East Anatolian and South Anatolian Red cattle—TT, CT, and CC. Furthermore, Pasandideh et al[34] identified 3 genotypes—CC, CT, and TT—of the *OPN* gene c.8514C>T in Iranian Holstein cows. The polymorphism of *OPN* is evaluated in Iranian Holstein Bulls and is noticeable in 3 genotypes—TT, CT, and CC.[35] Kułaj et al[36] detected the polymorphism of the *OPN* gene in Holstein-Friesian cattle and determined 3 genotypes—CC, CT, and TT—in the c.8514C>T locus. To the best of our knowledge, no literature is available on the polymorphism of *OPN* in Awassi sheep. This research addresses this gap by providing genotypic information and reporting new associations that may be useful when selecting sheep. Future use of marker-assisted selection for measuring economic traits may also be possible.

The study results showed a significant association ($P \leq .01$) between p.Q>R234 and prolificacy with the CC genotype with lower litter size, twinning rate, lambing rate, and more days to lambing than with the TC and TT genotypes. Therefore, the c.137T>C SNP mutation negatively affected these traits. Osteopontin plays a critical role in reproduction as

a fertility protein.[12] During pregnancy, OPN/SPP1 is synthesized and secreted by the glandular epithelium of the uterus as well as the luminal epithelium, where it stimulates nutrient transit by binding to integrins, facilitating the transfer of nutrients from the chorionic membrane to the placental vasculature and allantoic cavity of the embryo/fetus.[37] By attaching to specific integrins, OPN facilitates trophectoderm differentiation and attachment to the uterine luminal epithelium, which may play a crucial role in conceptus expansion and implantation.[38] In addition, OPN is vital for the development of reproductive functions and placental efficiency.[39] According to studies conducted on sheep,[40] swine,[41] and goats,[14] placental efficiency has a higher heritability and a significant positive correlation with litter size. There is a significant association between OPN polymorphisms and litter size, litter, and placenta weight in Dazu Black and Lezhi Black goats, suggesting that OPN is a suitable gene for determining litter size. Zhao et al[14] found that Dazu Black does with genotype AA had 0.88 more litters than Dazu Black does with genotype AB. In pigs, the *OPN* gene has been found to correspond to the location of Quantitative trait loci (QTL) regions for reproductive traits, including ovulation rate, litter size, and embryo survival.[42] Kumchoo and Mekchay[16] identified 3 SNPs that were associated with litter size in Thai large white pigs (c.425G>A, c.573T>C, and c.881C>T). Recently, there seems to be a significant relationship between purebred sows carrying the OPNe6-462 and OPNp3-617 genotypes and piglet size and the number of weaned piglets.[17] Numerous in silico analyses confirmed the damaging effects of the p.Q>R234 SNP on the structure, biological functions, and stability of OPN; for example, a deleterious nsSNP may adversely affect OPN's ability to perform its function. Consequently, the greater frequency of p.Q>R234 results in damaged OPN synthesis leading to lower prolificacy of Awassi ewes. Moreover, our results revealed a high rate of CC genotypes in single-producing ewes. In this genotype, a SNP with p.Q>R234 has a significant damaging effect on OPN. Several well-known fecundity genes have been studied in Awassi sheep, but the sheep are still prone to low fertility. It is thus necessary to investigate all possible unstudied genes to determine their effect on sheep fertility.[4,6,43] These results suggest that the p.Q>R234 SNP is one of the causal factors for the lower prolificacy of twin-producing ewes observed in these populations. Based on the available evidence, *OPN* is a promising candidate gene for animal reproduction. However, no further analysis of *OPN* has been conducted on Awassi breeds with different reproductive performances.

Conclusion

A novel SNP, p.Q>R234, was detected in the TC genotype of the *OPN* gene (exon 7). Association analysis between p.Q>R234 SNP locus and prolificacy showed that ewes with the CC genotype had significantly lower litter sizes, twinning and lambing rates, and more days to lambing than their TT and TC genotypes. In comparison with single-producing ewes with CC genotypes, twin-producing ewes with TT genotypes showed greater measured prolificacy. Based on the results of this study, it is evident that ewes with the CC genotype are negatively associated with prolificacy. The findings of this article strongly suggest excluding ewes that have p.Q>R234 SNPs from studies that require high reproductive performance values.

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Author Contributions

All authors contributed equally.

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