A Novel p.K116Q SNP in the OLR1 Gene and Its Relation to Fecundity in Awassi Ewes

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Research

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Abstract

Background

Sheep's fecundity is determined by both twinning rate and litter size, both influenced by several genes, one of which is *OLR1* (oxidized low-density lipoprotein receptor gene). This study aimed to determine the genetic variation of the *OLR1* gene affecting the fecundity traits of Awassi ewes.

Results

Two genotypes of 334-bp amplicons, CC and CA were detected. In a sequence reaction, the novel mutation p.K116Q was discovered in CA genotypes. There was a highly significant ($P \leq 0.01$) association between the single nucleotide polymorphism (SNP) and reproductive traits, in that sheep with the p.K116Q SNP had lower litter size, twinning rate, fecundity, and lambing percentages than individuals with CC genotypes. The ewes with CC genotypes had 1.98 lambs more than those with CA genotypes. These observations imply that the missense p.K116Q variant has an adverse effect on the traits under study.

Conclusions

As such, SNP p.K116Q appears to negatively influence fecundity traits in Awassi sheep. The data evidence that the *OLR1* gene variant is one of the most important candidates for marker-assisted selection in the sheep industry.

Background

The fecundity in sheep flocks is an important determinant of profitability [1]. A sheep's fecundity is determined by twinning rate and litter size, which are affected by many different genes [2]. One of these is the oxidized low-density lipoprotein receptor gene (*OLR1*) [3] that resides in chromosome 3 in sheep and contains 6 exons (Adopted from ncbi.nlm.nih.gov); in chromosome 5 in cattle with 5 exons [4]. This gene encodes OLR1 protein and belongs to the C-type lectin family that functions to absorb and degrade low-density lipoproteins [5], which impacts energy balance, in turn influencing reproductive characteristics [3]. It is also possible that this encoded protein functions to regulate fecundity genes so that the *OLR1* gene is involved in sheep fecundity [2].

Several studies have been reported the polymorphism of the *OLR1* gene in livestock and its relationship to phenotypic traits. An SNP within the exon and intron regions of the *OLR1* gene is associated with phenotypic traits in cattle, according to Komisarek and Dorynek [6] and Fonseca *et al*. [7]. Furthermore, *OLR1* is regarded as a potentially important gene in dairy cattle growth traits [8, 9]. The 3' untranslated regions of *OLR1* (NW_215807: g. 8238C>A) contain a polymorphism associated with the fat content of milk [10]. Besides, the SNP in the bovine *OLR1* gene (c. 495 T> C) affects carcass traits, fat accumulation, and growth in Angus, Charolais, and hybrid cattle [11]. The *OLR* gene is investigated in another study conducted on Dutch Holsteins/Friesians; the results indicated that *OLR* g.8232C>A significantly affected ($P < 0.05$) the fat content of milk [12]. A genetic variant in buffalo *OLR1* at position 8,232 in the 3'-UTR has been affected by fat content and yield in the Mehsana breed [13]. Additionally, Javed *et al*. [14] provided evidence of the association between the polymorphisms of the *OLR1* gene and growth traits, as well as dairy quality parameters in buffalo. The A/C SNP in the 3'-UTR fragment of the *OLR1* gene has been linked to lactational traits in Iranian Holstein cattle [15]. According to a recent study, the bovine *OLR1* T10588C and T10647T mutations have significant associations with fat distribution, back fat thickness, and intramuscular fat accumulation [16]. Additionally, there is a relationship between *OLR1* gene genetic polymorphism and litter size in Xidu black pigs. The TT genotype exhibited significantly larger litter sizes in comparison to TC and CC genotypes ($P< 0.01$), indicating that the 7-bp mutation T/C at intron 4 had a significant effect on litter size traits [17]. Considering the aforementioned studies, there has been little research conducted on the impact of genetic polymorphisms in the *OLR1* gene on fecundity traits in livestock, and no research on their association with fecundity traits in Awassi sheep.

Methods

Animal

Two hundred mature ewes (114 with single progeny and 86 with twins) were used in this study, not pregnant or lactating, and aged between 2.5 and 5 years. Two herding stations (Babylon and Karbala, Iraq) were randomly selected to receive the animals. For the entire year, both grass and concentrated food (2.5 percent of their body weight every day, composed of barley, bran, salt (59%), (40%) and (1%) concentrates, respectively), as well as freshwater, were provided to the animals. Several fecundity traits were recorded at the stations,

such as twinning rate, lambing percentage, and litter size. Litter size was determined by dividing the number of lambs born by the number of ewes lambing.

DNA and PCR

In the morning, before feeding the sheep, blood samples were drawn from the jugular vein. A vacutainer tube containing EDTA was used to collect blood for genetic analysis. To extract genomic DNA, a rapid salting-out technique was employed [18]. The amplification of three different regions of the genetic code of *OLR1* was achieved using NCBI Primer-BLAST [19], provided by Bioneer (South Korea). A PCR experiment was conducted using Bioneer's PCR premix (50 µM, 10mM, 30mM, 1.5mM for dNTPs, Tris-HCl, KCl, MgCl2, and 1 U Top DNA polymerase). Using the thermal gradient device (Eppendorf, Germany), the best PCR amplification conditions were determined (Table 1). The denaturation was carried out for 4 minutes at 94 °C, followed by 30 cycles of denaturation for 30 seconds, annealing for 45 seconds, and elongation for 30 seconds. Accordingly, the results were verified by electrophoresis of PCR products on agarose gels (1.5%) and determining the gel image with a Chemidoc Gel Imager (Bio-Rad, USA).

SSCP (single-strand conformation polymorphism)

All PCR products were genotyped according to the protocol of Imran *et al*. [20]. The denaturing-loading buffer (95%, formamide, 0.05% xylene cyanol, and 20 mM EDTA, pH 8) was added to equal volumes for each PCR amplicon. Following 7 minutes of denaturation, the PCR amplicons were transferred onto wet ice and stored for 10 minutes. Using a 0.5 TBE buffer, samples denatured in neutral polyacrylamide gels were loaded on. Subsequently, the gels were electrophoresed for 4 hours at 200mA and 100V at room temperature. To stain the gels, the rapid staining protocol developed by Byun *et al*. [21] was used.

DNA sequencing and in silico analyses

Following the detection of SSCP bands in polyacrylamide gels, downstream reactions were performed using the methods of sequencing laboratories (Macrogen, Geumchen, Korea). A referring sequence of the *OLR1* gene was retrieved from NCBI's website (https://www.ncbi.nlm.nih.gov). A BioEdit ver, 7.1. was used to edit DNA polymorphisms within each detected genotype (DNASTAR, Madison) and visualized using SnapGene Viewer ver. 4.0.4 (http://www.snapgene.com). Amino acid reading frames were detected using the Expasy software [22]. Moreover, a comparison of amino acid sequences was conducted, using UniProtKB, with their corresponding sequences in the OLR1 database (http://www.uniprot.org/align/). Many computational tools were used to predict the structure and function of mutant proteins, including SIFT [23], PolyPhen-2 [24], Provean [25], Panther [26], and PhD SNP [27].

Statistical analyses

Genotype and allele frequency were analyzed by PopGen32, version 1.31 [28]. Using Hardy-Weinberg disequilibrium law, disequilibrium was calculated. According to Botstein *et al*. [29], polymorphism information content (PIC) was computed. Association analysis of the *OLR1* genotypes was performed using IBM SPSS 23.0 (NY, USA), with a general linear model as follows:

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

Where: Y_{ijkl} = phenotype characteristics, µ = the mean of all traits, G_i = fixed effect of i^{th} genotypes (i = CC, AC, AA) P_j = fixed effect of j^{th} parity (j = 1, 2, 3, 4), A_k = fixed effect of k^{th} age group (2.5,>2.5-3.5,>3.5-4.5,>4.5-5), and e_{ijkl} = random residual error. Tukey-Krammer tests were performed to examine differences between means with a level of significance of ($P \leq 0.05$). Reproductive traits of fecundity (the number of lambs weaned per ewe), lambing percentage (the number of lambs born per ewe lambed), and ewe birth type were analyzed using Chi-square test. Based on preliminary analysis, effects of interaction and lambing season were unaffected by the model findings and were excluded.

Results

Genotyping of *OLR1* gene

The PCR amplification of three genetic fragments of 334 bp, 291 bp, and 274 bp was performed on the 3 *OLR1* coding regions, along with their flanking regions (Fig. 1, A). Although the 291 bp amplification of exon 4 by PCR was designed, PCR experiments turned out to be problematic since agarose gels showed no specific bands. Following this, PCR-SSCP patterns were designed to cover exon 6 exhibited monomorphic electrophoretic migration and no heterogeneity was observed. Furthermore, two distinctive PCR-SSCP patterns

were observed in the 334 bp amplicons designed for exon 3 (Fig. 1, B). The sequencing results showed that only one of the SSCP variants carried the C246A SNP, indicating exon 3 heterogeneity. Based on the C246A substitution, the detected SSCP variants were given genotypic values CC for homozygous C/C SSCP variants and CA for heterozygous C/A SSCP variants present at position 246 of PCR amplicons (Fig. 1, C). The Expasy software indicated that a missense (p.K116Q) occurred at the 116th position of the mature OLR1, where lysine (Lys) was exchanged with glutamine (Glu) (Fig. 1, D). Regarding the genetic diversity of p.K116Q, the genotype CA was predominant with a total frequency of 0.75 (n = 149) and a lower prevalence for the genotype CC with a total frequency of 0.25 (n = 51) (Table 2). However, the Chi-square test showed that polymorphism in the *OLR1* gene at the K116Q SNP locus deviated significantly from the HWE ($P \leq 0.05$).

In silico prediction of p.K116Q

All used *in silico* prediction tools were given neutral/non-deleterious signals for p.K116Q. The I-Mutant 2 tool further confirmed that the p.K112Q SNP does not function deleteriously, as it predicted increased stability of OLR1 upon modification of this SNP (Table 3). This non-deleterious effect on the structure, function, and stability of the analyzed protein may be attributed to the noncritical positions that it occupies on the OLR1 protein.

OLR1 gene analysis: statistical results

As such, based on the association analysis of p.K116Q SNP locus with fecundity traits, individuals with CA genotype had significantly ($P \leq 0.01$) lower litter size, twinning rate, fecundity, and lambing percentage than individuals with CC genotype (Table 4). In comparison to that of CA genotype ewes, CC ewes had 1.98 lambs. Hence, the missense p.K116Q SNP had a negative impact on these traits.

Discussion

Polymorphism of *OLR1* gene and genetic diversity

The genetic diversity analysis of the *OLR1* gene in Awassi ewes revealed that genotype CA was predominant with a total frequency of 0.75 (n = 149) and a lower prevalence for the genotype CC with a total frequency of 0.25 (n = 51). However, the Chi-square test showed that polymorphism in the *OLR1* gene at the K116Q SNP locus deviated significantly from the HWE ($P \leq 0.05$). Numerous studies have reported polymorphisms in the *OLR1* gene in livestock in various regions. In direct genomic sequencing of *OLR1*, Khatib *et al*. [8] identified 3 genotypes CC, AC, and AA in Holstein Dairy Cattle with 3'-UTR polymorphism. Using PstI/PCR-RFLP, Komisarek and Dorynek [6] identified 3 genotypes of *OLR1* in Polish Holstein-Friesian bulls CC, CT, and TT. Further, Javed *et al*. [14] revealed the polymorphism in exonic regions of the *OLR1* in Nili Ravi buffalo and determined 3 genotypes AA, AB and BB. Furthermore, a single nucleotide polymorphism (SNP) in the promoter region of the *OLR1* gene (c.-495 T>C) in hybrid, Angus, and Charolais beef cattle is identified and assigned CC, CT, and TT genotypes [11]. Fonseca *et al*. [7] genotyped rs109019599 in *OLR1* by PCR-RFLP assays and observed in the agarose gel AA (254 bp), CC (269 bp), and AC (269 and 254 bp). Moreover, the identification of *OLR1* genotypes in the 3’UTR region of dairy cattle is performed using PCR-RFLP, i.e., AA, AC and CC genotypes [3]. Recently, Anggraeni [4] identified the base mutations of the *OLR1* gene at 3'UTR location by PCR-RFLP techniques in Holstein Friesian dairy cattle and found 3 genotypes CC, AC, and AA. The aforementioned studies indicated that no studies have been undertaken on *OLR1* genotyping in Awassi sheep. Therefore, this study provides genotypic information and new associations that may prove more useful in selecting sheep and be recommended for measuring functional traits in future marker-assisted selection programs.

Sequencing and *in silico* analyses of *OLR1* gene

By detecting the C246A SNP in only one of the SSCP mutations, a sequencing reaction confirmed the presence of heterogeneity in exon 3 (Fig. 1, C). A p.K116Q SNP was positioned in the 116th lysine residue of the mature OLR1, where an amino acid substitution occurred from lysine (Lys) to glutamine (Glu) (Fig. 1, D). Some SNPs occur in non-coding regions, whereas others in coding regions [30]. These non-synonymous SNPs modify amino acid sequences when present in a coding sequence (called mutant SNPs), altering protein activity and causing drastic phenotypic changes [31, 32]. The missense mutation can negatively impact function not only by changing the protein's stability but also by interfering with the structure and function of other biological molecules [33]. Additionally, missense mutations can affect a molecule's flexibility and alter its equilibrium or alter the dynamics of its conformation [34]. All the *in silico* prediction tools used in the current study provided neutral or non-deleted signals for p.K116Q (Table 3). This non-deleterious effect in terms of the structural, functional, and structural stability of the analyzed protein occurs due to its non-critical positions where occupied

in this protein. Studer *et al.* [35] note that mutations can change an organism's phenotype in any way, perhaps with a beneficial, detrimental or neutral effect on its fitness.

Association analyses of *OLR1* gene with fecundity traits

This study examined the association between p. K116Q SNP locus and fecundity traits and revealed that individuals with AC genotype had significantly ($P \leq 0.01$) lower litter size, twinning rate, fecundity, and lambing percentage compared to CC genotypes (Table 4). As a result, these traits were negatively affected by C246A SNP mutation. In this regard, only one article has addressed the potential association between polymorphisms in the porcine *OLR1* gene and litter size. This study showed a T/C mutation at 7bp of intron4 with 3 genotypes TT, TC, and CC. The litter size of TT genotyped piglets is significantly higher than that of TC and CC genotyped piglets [36].

Importantly, the OLR1 degrades the oxidized forms of low-density lipoproteins (ox-LDL) and promotes cholesterol accumulation and adipocyte fatty acid uptake, thereby affecting liver fat metabolism [3, 6]. Although these roles are significant, changes in the lipid profile or fat content could lead to clinical and physiological problems [37]. Thus, by upregulating OLR1, adipocytes increase their total cholesterol content and capacity to absorb free fatty acids (FFA). If the adipocyte's lipid storage capacity is exceeded, it becomes unable to control the release of FFAs into the blood circulation, ultimately resulting in abnormal accumulation of lipid outside the adipocyte [38]. Moreover, excess fatty acids in the ovary generate damage to the cumulus and granulosa cells responsible for normal steroidogenesis in the gonads [39]. Furthermore, the accumulation of intracellular lipids causes high levels of free fatty acids to form, leading to oxidative damage and the production of highly active oxidative metabolites that cause irreversible cellular damage [40]. It has been shown that these metabolic changes can damage oocytes directly or by affecting their follicular environment [41]. Based on these data, *OLR1* gene variation causes several damaging aspects on oocytes, which could adversely affect fecundity traits. Consequently, the Awassi breed is characterized by a high level of adaptability to harsh environments [42] with a lower reproductive capacity [43, 44]. The low reproductive rate of this breed is a major concern for many breeders in the Middle East. Further, the low prevalence of the CC genotype with a total frequency of 0.25 (n = 51) in this study is likely to be one of several factors contributing to the low fertility of the Awassi breed.

Conclusion

A novel single nucleotide polymorphism (SNP), p. K116Q, was found in the *OLR1* gene (exon 3), in the heterozygous CA genotype. The results of the association analysis between p. K116Q SNP locus and fecundity traits revealed that individuals with the CA genotype had significantly lower litter sizes, ewe birth types, fecundity, and lambing percentages than those with the CC genotype. Twin-producing ewes with CC genotypes showed more measured fecundity traits than single-producing ewes with CC genotypes. In light of these results, *OLR1* is a promising candidate gene for sheep marker selection.

Declarations

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Author contribution

Tahreer M. Al-Thuwaini: Conceptualization, writing-original draft, supervision, methodology (lead). **Mohammed Baqur S. Al-Shuhaib**: Editing, data curation, and investigation (lead). **Mohammed M. Mohammed**: Methodology (equal).

Data and model availability statement

None of the data were deposited in an official repository but data are available upon request.

Ethics approval

With approval no. 020,7,18 provided by Al-Qasim Green University, this study was conducted from July 2020 to March 2021 and followed the international guidelines for the veterinary care and use of livestock.

Consent for publication

Not applicable.

Conflict of interest statement

None.

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Tables

Table 1. The oligonucleotide primer sets designed for the amplification of the ovine *OLR1* gene. The symbols "F" and "R" refer to forward and reverse primers, respectively. The design was based on the ovine NCBI Reference Sequence NC_040254.1.

Primer code	Locus	Sequence (5′ - 3′)	Binding coordinate in the genome		Amplicon length	Annealing temperature
			Start	Stop		
OLR1,exo3-F	Exon 3	TTCACCGAAATCCAGTGGAGG	220107792	220107812	334 bp	60.4 °C
OLR1,exo3-R		CCTCTCGCCCTCATACCTGA	220108106	220108125		
OLR1,exo4-F	Exon 4	AGAGATGGCATGCATGTGGG	220113177	220113196	291 bp	NA*
OLR1,exo4-R		AATCCAGCCTGACACATCCC	220113448	220113467		
OLR1-exo6-F	Exon 6	CCCTGCCTGCAGGTTTAGAA	220114431	220114450	274 bp	59.1 °C
OLR1-exo6-R		CTAGTAGCAGGCAGTTCCCG	220114685	220114704		

* No specific results were obtained from utilizing this primers' pair to amplify this locus since no specific bands (~ 291 bp) were observed in agarose gel electrophoresis.

Table 2. Genetic diversity of the *OLR1* gene in Awassi ewes.

Observed genotypes		Genotype frequencies		Allele frequencies		*Ho*	*He*	*Ne*	PIC	χ^2
CC	CA	CC	CA	A	C					
n= 51	*n*= 149	0.25	0.75	0.63	0.37	0.74	0.47	1.87	0.35	69.93

Abbreviations: χ^2 – Chi-square, *Ho* – observed heterozygosity, *He* – Expected heterozygosity, *Ne* – effective allele frequency, *n* – number of individuals. All Chi-square tests have one degree of freedom and within the significance level P≤ 0.05.

Table 3. The *in silico* prediction of p.K116Q on ovine OLR1 protein, in terms of structure and function.

SIFT		Meta-SNP		PROVEAN		SNAP		PhD-SNP		I-Mutant2	
Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction
0.130	Neutral	0.147	Neutral	-0.97	Neutral	0.260	Neutral	0.153	Neutral	1.44	Increase

SIFT: Value > 0.05 mutation is predicted neutral; Meta-SNP: Value < 0.5 mutation is predicted neutral; PROVEAN: value > -2.5 is predicted neutral; SNAP: Value < 0.5 mutation is predicted neutral; PhD-SNP: Value < 0.5 mutation is predicted neutral; I-Mutant2: DDG > 0

increase stability.

Table 4. The association between *OLR1* genetic polymorphism at locus p.116K>Q and fecundity traits in Awassi ewes.

Genotypes	Litter size (LSM ± SE)	Ewe birth type (%)		Fecundity	Lambing percentage
		Singleton	Twin		
CC (51)	1.98± 0.04[a]	12(23.52%) [b]	39(76.47%) [a]	1.76 [a]	176.47 [a]
CA (149)	1.42± 0.05 [b]	102(68.45%) [a]	47(31.54%) [b]	1.31 [b]	131.54 [b]
P-value	0.01*	0.01*	0.04*	0.02*	0.03*

LSM ± SE, Least square means ± Standard error, * (P≤0.05). [a,b] Significant differences in means represent by differences in the same column within each classification.

Figures

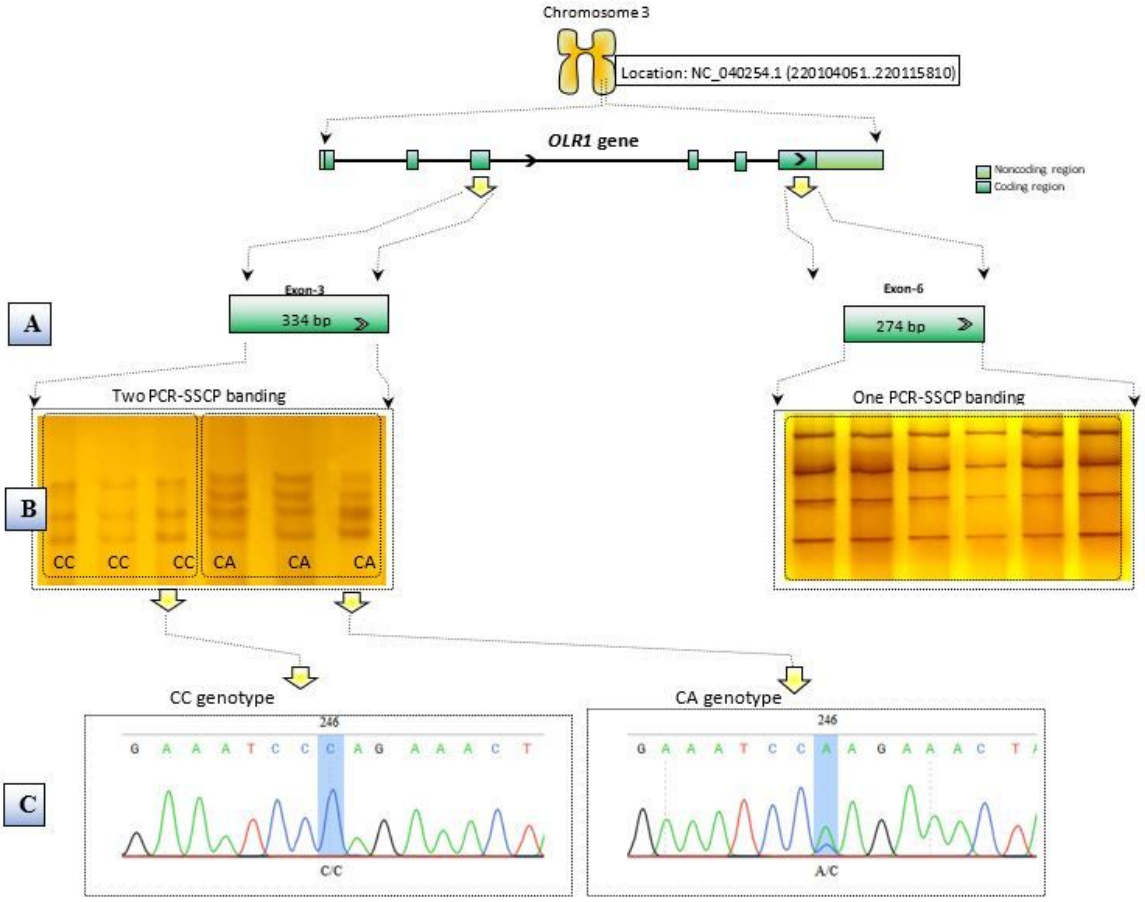

Figure 1

An overview of the OLR1 gene-based PCR-SSCP-sequencing method in Awassi ewes. A) Design of two distinct PCR primer pairs encoding sequences measuring 334 and 274 bases respectively within exon 3 and exon 6. B) Only one exon 3 was identified as homozygous and heterozygous based on PCR-SSCP genotyping. C) Electropherograms of DNA sequencing data showing that one SNP, C246A, was located in a heterozygous CA genotype at exon 3.