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Case–Control Possible Association between Congenital Supernumerary Extra Functional Teats and IGF1, Leptin, Calpastatin and FBP30 Candidate Genes in Fat-Tailed Ghezel Ewes

Research Article

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ABSTRACT

Supernumerary teats (SNT) are a prevalent and intricate polygenic trait that is frequently observed at birth in sheep. These additional teats have been linked to a heritable trait associated with maternal capability, especially in ewes with high fertility. Investigating the genomic basis of these characteristics is an ongoing endeavor and poses an intriguing research inquiry with implications for both biological understanding and practical applications. In order to delve into this scientific enigma, a specific cohort of 20 local fat-tailed Ghezel ewes, each possessing four functional teats, was carefully chosen to form the case group. In parallel, a control group comprising 25 healthy ewes with the standard two normal teats was established for comparative analysis. Blood samples were meticulously collected from both groups of Ghezel ewes demonstrating distinct phenotypic expressions, specifically those with either two or four functional teats. Following the sample collection, the genomic DNA extraction process was carried out using a commercial DNA purification kit provided by Qiagen company, known for its high-quality DNA extraction products. We conducted an examination of four PCR-RFLP markers on four candidate genes (IGF1, Leptin, Calpastatin, and FBP30). Through our research, we unveiled the allele distribution and genotype frequency of these potential genes in both the case and control groups of sheep. Our findings demonstrated notable disparities in the genotype and gene frequencies between the case and control groups for each candidate gene. Furthermore, we calculated the odds ratio (OR) and 95 percent confidence intervals (CIs) under Hardy-Weinberg disequilibrium using various genetic models. The $\chi 2$ test was also utilized to compare the genotype and allele distribution of these candidate gene polymorphisms between the case and control groups. The results of our study are particularly intriguing as they indicate that individuals with an AA genotype for the leptin gene are significantly more likely to have four additional functional teats (OR=7.7143, 95 percent CI=1.6025, P-value=0.0108). Additionally, the results in a dominant model (AA *vs*. GA+GG) revealed an OR of 0.1296 and 95 percent CI of 0.02669–0.6240, with a p-value of 0.0108, further highlighting the association between leptin genotypes and supernumerary teats (SNT). Moreover, the risk of having more than four functional teats is heightened by the GG genotype in the FBP3 gene ($P<0.13$). However, our analysis did not uncover any additional significant correlation between the polymorphism of another investigated candidate gene and supernumerary teats (SNT). Overall, this topic presents an intriguing area that warrants further research to gain a comprehensive understanding of the biological mechanisms governing the development of four functional teats in ewes with large litter sizes.

KEY WORDS candidate gene, IGF1, mammary gland, PCR-RFLP, sheep, supernumerary teat.

INTRODUCTION

Supernumerary teats (SNT), also known as polythelia, are a common anomaly in many mammals, including sheep, as indicated by recent theoretical developments (Kenny *et al.* 2014). This phenomenon is generally considered undesirable due to its potential negative impact on milking efficiency, udder damage, and prolonged milking duration in normal sheep families (Kenny *et al.* 2014; Butty *et al.* 2016; Martine *et al.* 2016; Zhang *et al.* 2019).

The presence of supernumerary teats (SNT) in sheep and goats can be fully functional for milk production and may offer a solution for dealing with orphaned lambs. Research suggests that creating synthetic sheep breeds with four functional teats could be beneficial. The intra-uterine position of the fetus and prenatal hormones are likely causing of SNT (Kenny *et al.* 2014; Raheem *et al.* 2014; Martine *et al.* 2016). Ewes expressing four healthy and functional teats may be more effective for suckling lambs, potentially reducing production costs (Zhang *et al.* 2019; Hardwick *et al.* 2020). New research suggests that certain animal breeds are linked to having extra teats. This trait is associated with higher birth rates in sheep and goats, and is desirable in pigs. Further research is needed at the DNA level to understand the genetic basis of this trait (Lundeheim *et al.* 2013; Kenny *et al.* 2014; Martine *et al.* 2016; Peng *et al.* 2017).
The genes controlling sheep mammary gland development are not well known. Understanding these genes at the embryonic stage will help shed light on epithelial differentiation. The *IGF1* gene has been identified as associated with regulating lactation traits, mammary gland development, and ovarian follicle survival and maturation (Kenny *et al.* 2014). Other genes involved in abnormal teat development include WNT10A, identified through candidate gene approach and quantitative trait loci (QTL) analysis (Davenport *et al.* 2003; Chu *et al.* 2004). Mutations in the TBX3 gene, encoding T-box transcription factor 3 (Tbx3), have also been linked to the prevalence of SNT. Genes encoding fibroblast growth factor 10 (FGF10), FGF receptor 2B (FGFR2B), T-box transcription factor TBX3, and lymphoid enhancer factor 1 (LEF1) play important roles in mammary bud development and maintenance (Van Genderen *et al.* 1994; Veltmaat *et al.* 2004; Xu *et al.* 2014). Additionally, mammary mesenchymal tissue differentiation and nipple development depend on PTHrP and the type 1 parathyroid hormone/parathyroid hormone-related protein receptor (PTH1R) (Foley *et al.* 2001).

The *IGF1* gene is crucial for prenatal and postnatal growth and plays a role in regulating mammary gland development (Bakhtiar *et al.* 2017; Darwish *et al.* 2017). It is also linked to important traits in livestock animals. Leptin is essential for mammary gland development during fetal and newborn growth (He *et al.* 2012; Gobikrushanth *et al.* 2018). In general, the *IGF1* gene plays a crucial role in prenatal and postnatal growth. It is involved in regulating mammary gland development and has been linked to important traits in livestock animals. The *IGF1* gene varies in length and exon numbers across different species (Boucher *et al.* 2006). In sheep, the *IGF1* gene is located on chromosome 3 (Liu *et al.* 2010; Wang *et al.* 2011; Sharma *et al.* 2013; Darwish *et al.* 2017; Meira *et al.* 2019). Leptin has also been identified as essential for mammary gland development during fetal and newborn growth (Scatà *et al.* 2010).

During pregnancy and breastfeeding, the hormone leptin interacts with other hormones. The 16 kDa LEP gene is found on sheep chromosome 4. The calpains-calpastatin system is crucial for early activation of the mammary gland during weaning, with calpain-1 cells located in the core of nonepithelial breast tissue. The calpastatin gene, located on sheep chromosome 5, plays a role in biological processes in the mammary glands of dairy farms (Zhou *et al.* 2009; Lakhssassi *et al.* 2020). During pregnancy and breastfeeding, the hormone leptin interacts with other hormones. The 16 kDa LEP gene is found on sheep chromosome 4 (Javanmard *et al.* 2008; Grochowska *et al.* 2017; Pophiwa *et al.* 2020). The calpains-calpastatin system is crucial for early activation of the mammary gland during weaning, and the calpastatin gene, located on sheep chromosome 5, plays a role in biological processes in the mammary glands of dairy farms (Arnandis *et al.* 2012; Utsunomiya *et al.* 2013; Aali *et al.* 2017). Numerous studies have highlighted the important role of the small cytoplasmic protein known as fatty acid binding protein (FABP) in the growth of the mammary gland, particularly in the proliferation and growth of mammary epithelial cells and fatty acid metabolism during lactation (Calvo *et al.* 2004). FABP3, found on chromosome 2 in sheep, consists of four exons separated by three introns and has the same exon/intron structure (Liang *et al.* 2014). These results highlight the crucial regulatory role of leptin, IGF1, calpastatin, and FBP candidate genes in the development of the mammary gland and branchial alveolus in dairy animals. This study aimed to address the gap in the literature by comparing the effects of leptin, FBP3, calpastatin, and IGF1 on the SNT phenomenon in Ghezel fat-tailed sheep through a case-control study.

MATERIALS AND METHODS

Animals

Through widely used social media platforms, we extended invitations to sheep owners in northwest Iran to participate in our project. To thoroughly investigate the phenomenon in both breeds, we opted for a case study approach.

The case group included twelve local fat-tailed Ghezel ewes with four functional teats, while the control group comprised 25 healthy ewes with two normal teats. In Figure 1, we observed normal milk secretion from the mammary glands of ewes with four functioning abnormal teats exhibiting SNT.

Blood collection, DNA extraction and PCR-RFLP

In this experiment, we collected whole blood samples from two groups of Ghezel fat-tailed sheep – one with two functional, healthy teats and the other with four. The blood was drawn from the jugular vein using vacuum tubes containing the anticoagulant K_2-EDTA to prevent clotting. After collection, the blood samples were promptly stored in a -20 °C freezer to preserve their integrity and then transported to the laboratory on ice to maintain their quality during transit.

DNA extraction

We used a commercial DNA purification kit from Qiagen to extract genomic DNA. Following extraction, we carefully assessed both the quantity and quality of the DNA using low-melting agarose, ethidium bromide staining (0.05 g/mL), and horizontal electrophoresis. Our initial studies involved the examination of four PCR-RFLP markers on four candidate genes. For a comprehensive breakdown of the restriction enzyme, allele sizes, annealing temperature, amplicon size, and primer sequence, please refer to Table 1.

PCR-RFLP assay

Each specific primer was added in increments of 2 µL 0.5 mM, along with 2 U Taq polymerase (Denmark), 50 ng DNA template, PCR buffer (1X), 1 mM dNTP, and 2 µL 5 mM $MgCl_2$ to a total volume of 25 µL for the PCR reaction. RedSafe dye was used to stain the 2% low melting point agarose gels, and the results were observed under a UV lamp after 40 minutes of horizontal electrophoresis. The electrophoresis power supply model used was the PAC1000 manufactured by Bio-Rad in the USA. For estimating the size of the PCR product using a gel from Fermentas, a standard size marker ladder of 100 bp and 1 kb was used along with the BIO $1D^{++}$ computer program. PCR-RFLP genotyping was used to detect polymorphisms in the amplified region of the candidate genes.

Ten units of the appropriate restriction endonuclease-IGF1 (BstH2I), Leptin (Bcn1), Calpastatin (Msp I) and FBP3 (BseDI) (from New England Biolabs Ltd., Great Britain) were used to digest eight microliters of each PCR product at 37 °C for 14 hours. Following 90 minutes of electrophoresis at 120 V in 2% agarose gels, the resulting fragments were examined under a UV lamp.

The allele naming system used in this study was based on the original reports to provide relevant comparisons for discussion.

Statistical analysis

We employed POPGENE software (version 3.1) to conduct detailed molecular descriptive statistical analyses. This involved calculating the odds ratio (OR) and 95 percent confidence intervals (CIs) under Hardy-Weinberg disequilibrium using various genetic models. Additionally, we compared the genotype and allele distribution of these potential gene polymorphisms in the case and control groups using the $\chi 2$ test. We considered P-values less than 0.05 to be indicative of significance.

RESULTS AND DISCUSSION

The observed genotype pattern and the size of the RFLP alleles at the four loci examined were found to be in line with the original references obtained previously. Figure 2 presents a summary of the electrophoresis genotype size for the four candidate genes analyzed in Ghezel sheep.

Our study investigated the allele distribution and genotype frequency of four potential genes in both the case and control groups of sheep. We found notable differences in the genotype and gene frequencies between the case and control groups for each candidate gene. For detailed genotype and allele frequency values for the four candidate genes, please refer to Table 2.

A comprehensive summary of the data including odds ratios, 95 percent confidence intervals, P-values, and the results of the Hardy-Weinberg test for the four candidate gene polymorphisms examined in the SNT case group and controls is presented in Table 2.

The data in Table 1 indicates a significant increase in the incidence of having an additional four functional teats in individuals with the AA genotype (OR=7.7143, 95 percent CI=1.6025, P=0.0108). However, no significant difference was observed between the two groups for any of the other polymorphisms examined.

Table 3 highlights the association between leptin genotypes and SNT in a dominant model (AA *vs*. GA+GG) (OR=0.1296, 95 percent CI: 0.02669–0.6240, P-value=0.0108).

Despite this, our analysis did not reveal a statistically significant difference in the polymorphisms of other investigated candidate genes with SNT. Furthermore, no evidence of association between other candidate genes and the models examined was found between case and control subjects (Figure 2).

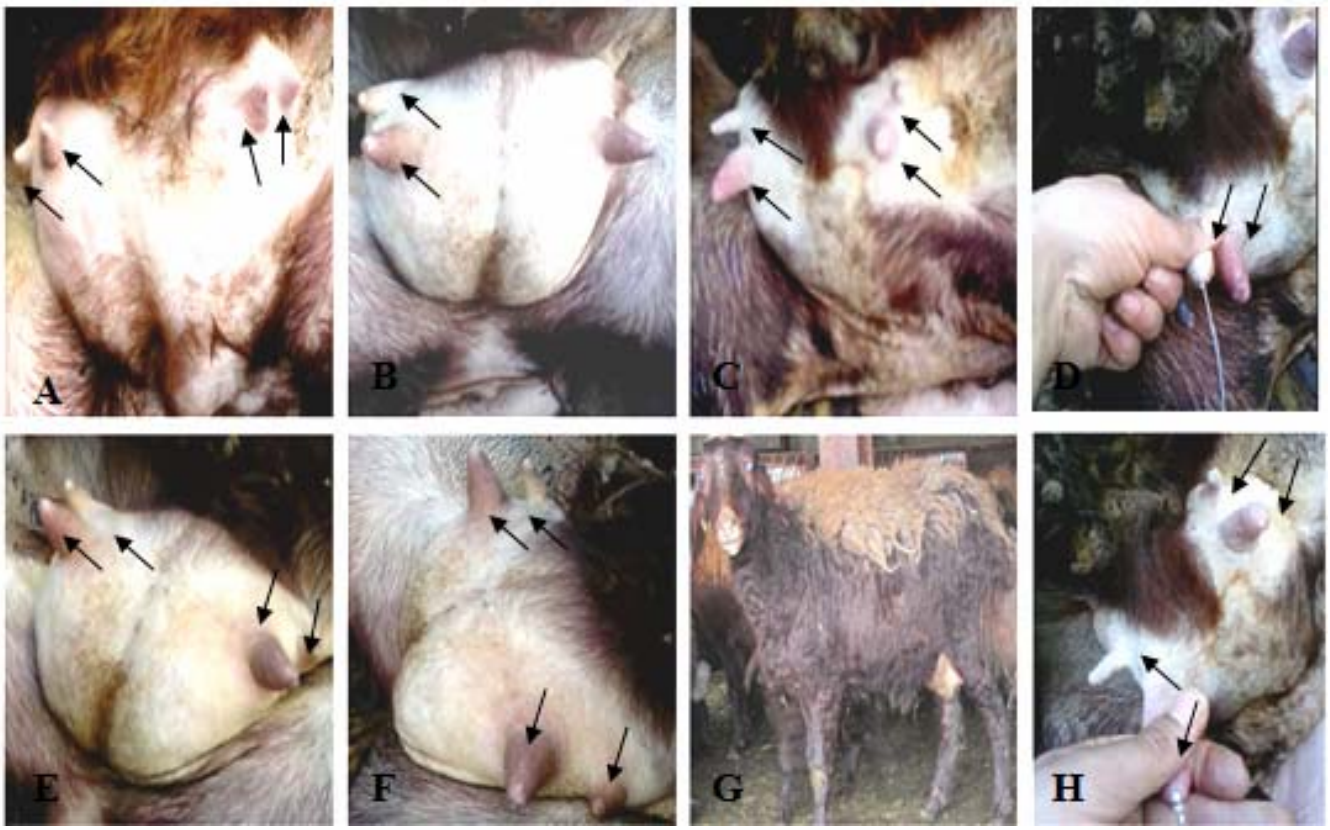

Figure 1 An illustration depicting supernumerary teats (SNT) in Ghezel ewes with four functional teats having normal milk secretion

Table 1 The primer sequences, annealing temperature, amplicon size, restriction enzyme, and allele sizes used in this study

Gene	Primer sequence	SNP	Annealing	RE	gene region	Fragments
IGF1 X17229	5′-ATTACAAAGCTGCCTGCCCCTT-3′ 5′-CACATCTGCTAATACACCTTACCCG-3′	G>A transition	61 °C	BstH2I	5' end and exon 1	265 bp 85 and 180 bp
Leptin	5′-AGGAAGCACCTCTACGCTC-3’ 5’-CTTCAAGGCTTCAGCACC-3’	G>A G 271 A	59 °C	Bcn1	Exon 3	471 270 201
Calpastatin	5′-TGGGGCCCAATGACGCCATCGATG-3 5-GGTGGAGCAGCACTTCTGATCACC-3	G>A	62 °C	Msp I	Exon 1 and intron	622 bp 336, 286
FBP3	5′-GGTTTTGCTACCAGGCAGGT-3′ 5′-TTCCCTATTCCCCTTCAGGG-3′	G>A	56 ◦C	BseDI.	Exon 2	222 bp 143, 43 and 36 bp

The nursing and milking ability of ewes with more than two litters in a parity could greatly benefit from an understanding of the genomic component of supernumerary teats (SNT) in sheep. Recent advances in molecular genetics allow us to uncover the genetic architecture of specific traits through the identification of individual genes or candidate genes. Our goal was to elucidate the genetic mechanism underlying SNT in Ghezel sheep to provide insights into this issue. The search for DNA biomarkers in candidate genes associated with the SNT code is an ongoing and crucial endeavor. The results of this study revealed significant differences in the frequency of four healthy teats between the breeds studied, ranging from 2 to 10%.

It's important to note that SNT can be functional (with milk secretion) or nonfunctional (no milk secretion); furthermore, SNT teats are not exclusive to females and can also be observed in males (Kenny *et al.* 2014).

Studies have shown that the presence of supernumerary teats (SNT) in livestock, particularly in goats, is highly heritable, and their physical removal after birth can reduce milk production significantly (Liang *et al.* 2014). In Turkish Saanen goats, approximately 17% tested positive for SNT (Martin *et al.* 2016), while SNT was found in 1.65% of the goat population in Bihar, India (Ozoje, 2002). Reports suggest that around 44% of goat populations suffer from SNT.

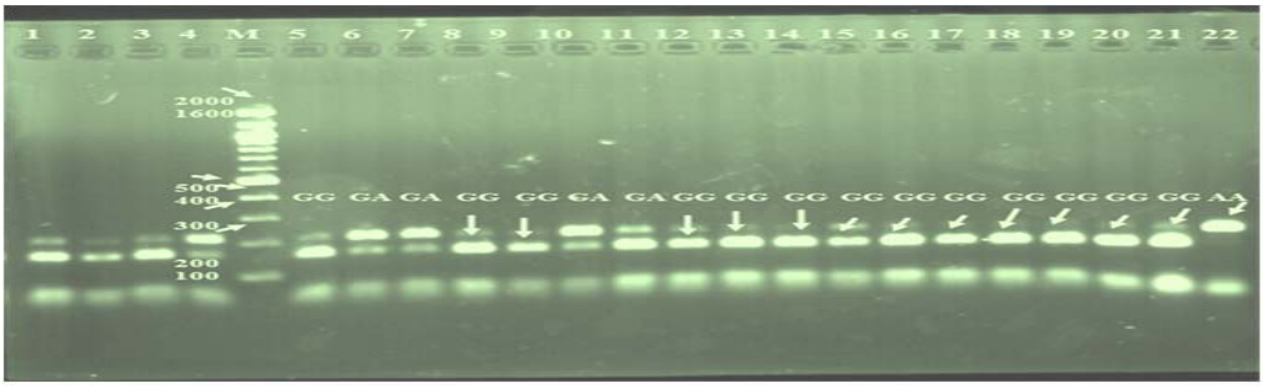

Figure 2(A)

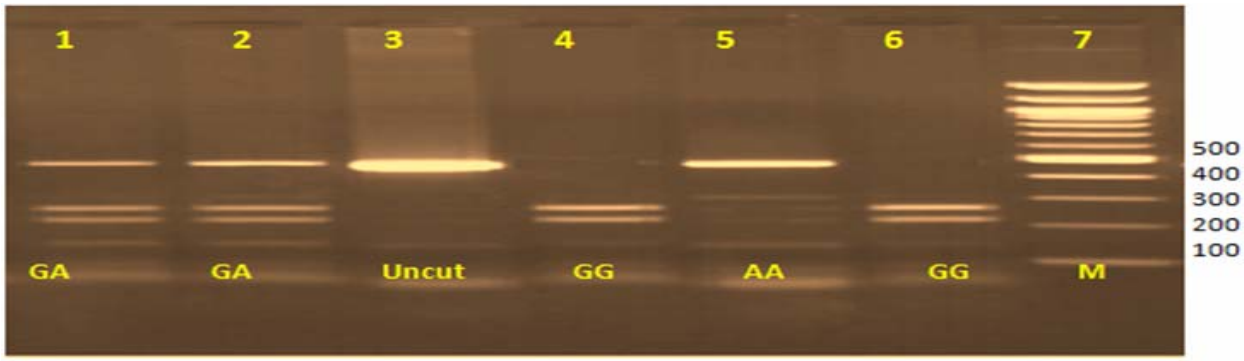

Figure 2(B)

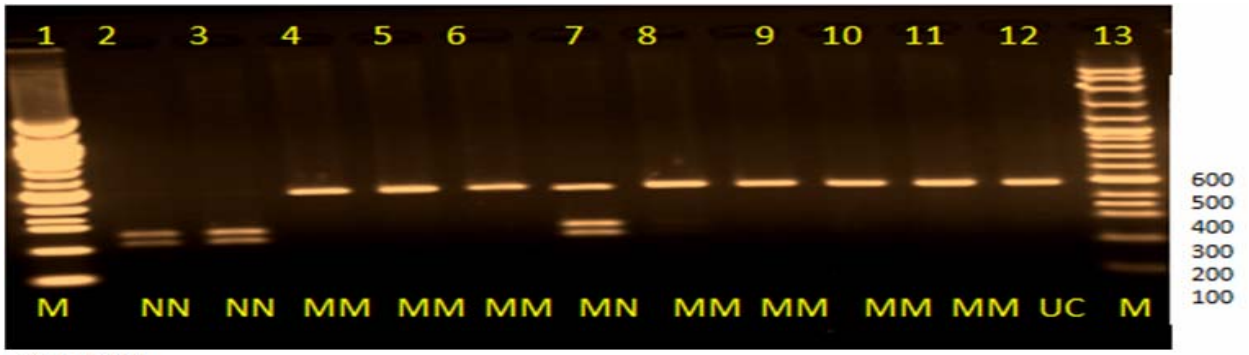

Figure 2(C)

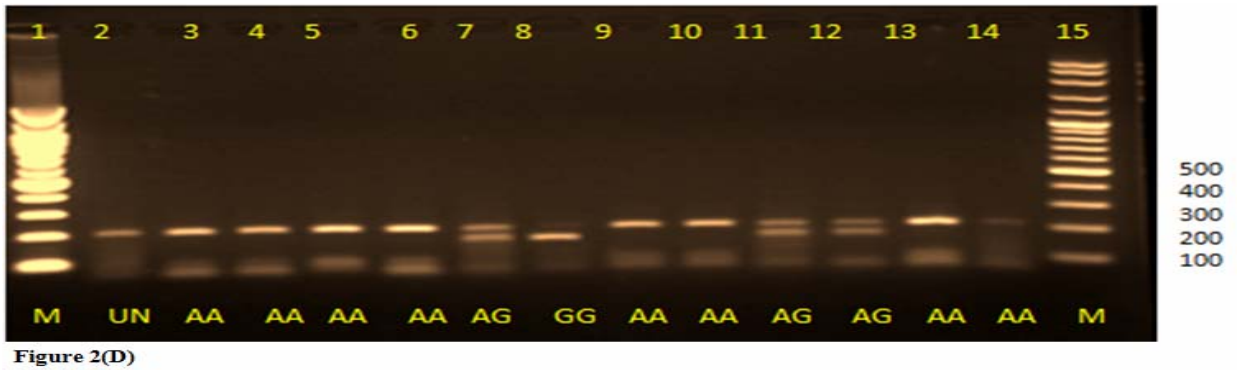

Figure 2(D)

Figure 2 The overview includes the RFLP pattern of the four studied candidate genes in this study

Note: 2(A): The RFLP pattern of IGF1 gene in this study. A 265-bp fragment of the IGF1 amplicon was digested with BstH2I to produce a 65 and a 100 bp fragments

2(B): The PCR-RFLP pattern of leptin gene. A 471 bp fragment was digested with Bcn1 restriction enzyme. The products of digestion were electrophoresed on 3% agarose and directly visualized with ethidium bromide under UV light. Lane M: 100 bp DNA ladder, lanes 1, 2, 6 and 8 are both chromosomes cut 270, 201 GG and lane 5 indicated uncut pattern AA genotype. **2(C):** The PCR-RFLP pattern of calpastatin gene. A 622 bp fragment was digested with Msp I restriction enzyme. The products of digestion were electrophoresed on 2% agarose and directly visualized with ethidium bromide under UV light. Lane M: 100 bp DNA ladder, lanes 1, 2, are undigested control and lanes 4, 5 are NN genotype (336 and 286 bp) both chromosome and lanes 6-8 indicated uncut pattern MM genotypes. **2(D):** The PCR-RFLP pattern of FBP3 gene. A 222 bp fragment was digested with BseDI restriction enzyme. The products of digestion were electrophoresed on 2.5% agarose and directly visualized with ethidium bromide under UV light. Lane M: 100 bp DNA ladder, lanes 2-6, are AA genotype (222 bp) and lanes 8 indicated cut pattern for both chromosome GG genotypes

Table 2 The overview includes comparisons of genotype and allele frequencies, odds ratios, 95% confidence intervals, P-values, and the Hardy-Weinberg test in the case-control groups

Gene ID	Item	Case n=12	Control n=25	Odd	95% CI	P-value	Z statistic
IGF1	GG	3 (0.25)	5 (0.20)	1.3333	0.2604 to 6.8279	0.7299	0.345
	GA	7 (0.58)	12 (0.48)	1.5167	0.3777 to 6.0906	0.5571	0.587
	AA	2 (0.17)	8 (0.32)	0.4250	0.0750 to 2.4099	0.3338	0.966
	G	13 (0.54)	22 (0.44)	2.7576	0.9652 to 7.8786	0.0582	1.894
	A	11 (0.46)	28 (0.56)				
	MAF	**A**	G				
	K-square	Chi=0.3668	Chi=0.0169				
Leptin	GG	1 (0.08)	10 (0.4)	0.1364	0.0151 to 1.2282	0.0756	1.777
	GA	2 (0.16)	8 (0.32)	0.4250	0.0750 to 2.4099	0.3338	0.966
	AA	9 (0.76)	7 (0.28)	**7.7143**	**1.6025 to 37.1354**	**0.0108**	**2.548**
	G	4 (0.16)	28 (0.56)	**0.1571**	**0.0469 to 0.5270**	**0.0027**	**2.997**
	A	20 (0.84)	22 (0.44)				
	MAF	**G**	A	**AA increase risk of supernumerary four functional teats**			
	K-square	Chi=1.9200	Chi=3.0739				
Calpastatin	NN	2 (0.16)	6 (0.24)	0.6333	0.1074 to 3.7335	0.6138	0.505
	NM	7 (0.58)	11 (0.44)	1.7818	0.4424 to 7.1760	0.4164	0.813
	MM	3 (0.26)	8 (0.32)	0.7083	0.1498 to 3.3492	0.6635	0.435
	N	11 (0.45)	23 (0.46)	0.9933	0.3740 to 2.6381	0.9893	0.013
	M	13 (0.55)	27 (0.54)				
	MAF	**N**	M				
	K-square	Chi=0.3668	Chi=0.3268				
FBP3	GG	8 (0.66)	6 (0.24)	**6.3333**	**1.3977 to 28.6974**	**0.0166**	**2.394**
	GA	4 (0.34)	14 (0.56)	0.3929	0.0934 to 1.6527	0.2025	1.275
	AA	0	5 (0.20)	0.1491	0.0076 to 2.9333	0.2106	1.252
	G	20 (0.83)	26 (0.52)	**4.6154**	**1.3785 to 15.4533**	**0.0131**	**2.481**
	A	4 (0.17)	24 (0.48)				
	MAF	**A**	A	**GG increase risk of supernumerary four functional teats**			
	K-square	Chi=0.4800	Chi=0.3708				

Additionally, a study on 589 dwarf goats from West Africa found that 7.3% had SNT (Bemji and Popoola, 2011).

In the West African Dwarf and Red Sokoto goat breeds native to Nigeria, SNT prevalence is considered a significant udder abnormality (Amao *et al*. 2003). Studies found that 5.3% and 64.3% of goats in the southern and southwest regions of Nigeria have extra teats (Famakinde *et al*. 2019). Our research examined functional teat distribution and their correlation with candidate genes for IGF1, leptin, calpastatin, and FBP3. PCR-RFLP results for each gene were consistent with types reported in the literature. Our study revealed allele distribution and genotype frequency of four potential genes in case and control groups of sheep, showing differences in genotype and gene frequencies between the groups for each candidate gene.

In our study on Ghezel sheep, there was no evidence that IGF1 polymorphism affected SNT. Previous research has emphasized the role of IGF1 in mammary gland development.

Our conclusions about genotype and allele frequency pattern align with studies on black Egyptian sheep (Darwish *et al.* 2017), which reported higher G allele frequencies (0.544) than A allele frequencies (0.456). Regarding the leptin gene, our results showed a greater distribution of the A allele than the G allele in both case-control groups. However, when comparing our results with previous studies on Malpura sheep, inconsistent patterns were observed, with only the monomorphic GG pattern reported. Additionally, a study discovered three SNPs in exon 3 of the leptin gene, leading to amino acid changes in several sheep breeds (Huang *et al.* 2008).

The genotype AA significantly increases the incidence of supernumerary four functional teats (OR=7.7143, 95 percent CI=1.6025, P=0.0108), which is a promising result. Leptin is essential for mammary gland development, and our observed results align with previous studies. However, reports of comparable associations between SNT and leptin polymorphism in cattle are lacking.

Table 3 The frequencies, odds ratios, and P-values of the four studied candidate gene polymorphisms among case-control subjects were analyzed under recessive and dominant models, as well as recessive and over-dominant heredity models

Gene	Model	Alleles	Case n=12	Control n=25	Odd	CI	P-value
IGF1	Dominant model	GA + GG	10 (0.83)	17 (0.68)	2.3529	0.4150 to 13.3422	0.3338
		AA	2 (0.17)	8 (0.32)	0.4250	0.0750 to 2.4099	0.3338
	Recessive model	GG	3 (0.25)	5 (0.20)	1.3333	0.2604 to 6.8279	0.7299
		AA + GA	9 (0.75)	20 (0.80)	0.7500	0.1465 to 3.8407	0.345
	Over dominant	GA	7 (0.58)	12 (0.48)	1.5167	0.3777 to 6.0906	0.587
		AA + GG	5 (0.42)	13 (0.52)	0.6593	0.1642 to 2.6478	0.5571
Leptin	Dominant model	GA + GG	3 (0.25)	18 (0.72)	**0.1296**	**0.0269 to 0.6240**	**0.0108**
	Recessive model	AA	9 (0.75)	7 (0.28)	**7.7143**	**1.6025 to 37.1354**	**0.0108**
		GG	1 (0.08)	10 (0.40)	0.1364	0.0151 to 1.2282	0.0756
	Over dominant	AA + GA	11 (0.92)	15 (0.60)	7.3333	0.8142 to 66.0497	0.0756
		GA	2 (0.16)	8 (0.32)	0.4250	0.0750 to 2.4099	0.3338
		AA + GG	10 (0.84)	17(0.68)	2.3529	0.4150 to 13.3422	0.3338
Calpastatin	Dominant model	MN + NN	9 (0.68)	17 (0.68)	1.4118	0.2986 to 6.6752	0.6635
	Recessive model	MM	3 (0.32)	8 (0.32)	0.7083	0.1498 to 3.3492	0.6635
		NN	2 (0.16)	6 (0.24)	0.6333	0.1074 to 3.7335	0.6138
	Over dominant	MM + MN	10 (0.84)	19 90.76)	1.5789	0.2678 to 9.3078	0.6138
		MN	7 (0.58)	11 (0.44)	1.7818	0.4424 to 7.1760	0.4164
		MM + MN	5 (0.42)	14 90.56)	0.5612	0.1394 to 2.2603	0.4164
FBP3	Dominant model	GA + GG	12 (1)	20 (0.80)	6.7073	0.3409 to 131.9637	0.2106
		AA	0	5 (0.20)	0.1491	0.0076 to 2.9333	0.2106
	Recessive model	**GG**	**8 (0.66)**	**6 (0.24)**	**6.3333**	**1.3977 to 28.6974**	**0.0166**
		AA + GA	4 (0.34)	19 (0.76)	0.5526	0.1562 to 1.9556	0.3577
	Over dominant	GA	4 (0.33)	14 (0.56)	0.3929	0.0934 to 1.6527	0.2025
		AA + GG	8 (0.67)	11 (0.44)	2.5455	0.6051 to 10.7087	0.2025

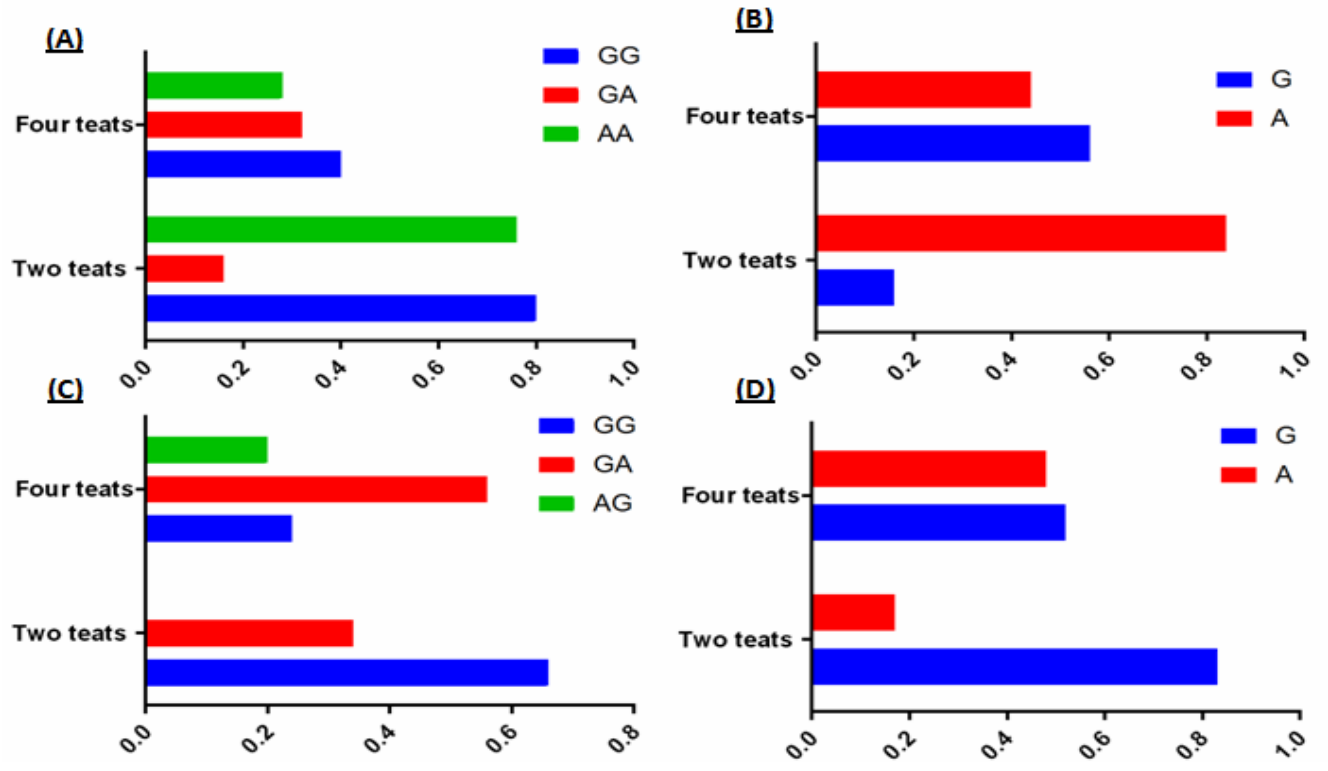

Figure 3 An overview of the genotype and allele frequency patterns among ewes with two teats versus four teats for the leptin gene **(A and B)** and the FBP3 gene **(C and D)** respectively: The AA genotype increases the risk of having supernumerary four functional teats in the leptin gene, and the GG genotype increases the risk of having supernumerary four functional teats in the FBP3 gene

Due to this barrier, we are unable to make comparisons with previous research. Additional candidate genes, such as relaxin1 (RLN1), growth hormone receptor, insulin-like growth factor, insulin-like growth factor 2, follicle-stimulating hormone beta, and growth hormone-releasing hormone receptor, have been highlighted in previous studies. However, no correlation between case and control subjects was found for the other candidate genes examined (IGF1, Calpastatin, and FBP3). Our results suggest variable genotype and gene frequencies for each candidate gene, consistent with related research. Reasons for this variability may include breed history, inbreeding level, animal species (beef or dairy), geographic location, sample size, risk of genotyping errors, and unmeasured variables.

Our results on the genotype and allele distribution of calpastatin are consistent with previous studies using the same PCR-RFLP methods, with sheep breeds showing varying N allele distribution. Similarly, our findings regarding the distribution of the FBP3-G allele align well with previous studies, such as those on Bulgarian sheep breeds showing similar PCR-RFLP and SNP patterns (Dimitrova *et al.* 2016).

These results align with studies indicating allele frequencies ranging from 0.13 to 0.23 for allele A and 0.77 to 0.87 for allele G in the FBP3 gene. Additionally, previous findings suggest that the mouflon, considered the ancestor of European sheep, exclusively produces offspring with the A allele. In domestic sheep, the frequency of allele A in the FBP3 gene varies, ranging from 0.26 in Raza Aragonesa to 0.46 in Manchega breeds, with the Turkish sheep breed Kıvırcık showing similar allele frequencies of 0.42 for allele A and 0.58 for allele G (Dimitrova *et al.* 2016).

The analysis was limited by the small sample size of four-teat ewes and the restricted number of candidate genes examined. Although the results are promising, further research using advanced technologies such as GWAS and next-generation sequencing is necessary to gain a deeper understanding of the genetic architecture of supernumerary teats and the presence of four functional teats.

CONCLUSION

The nursing and milking ability of ewes with more than two litters in a parity could greatly benefit from an understanding of the genomic component of supernumerary teats (SNT) in sheep. Our study aimed to elucidate the genetic mechanism underlying SNT in Ghezel sheep and identify DNA biomarkers associated with the SNT code. Our results indicate that the AA genotype for the leptin gene increases the likelihood of having four additional functional teats, while the GG genotype in the FBP3 gene increases the risk of having more than four functional teats.

However, our analysis did not reveal any further significant correlation between the polymorphism of another investigated candidate gene and SNT. These findings could confirm the crucial role of the leptin gene in the differentiation of the mammary epithelium during embryonic programming for future lactation. This study provides the first report of a potential gene contributing to congenital supernumerary four functional teats in thick-tailed Ghezel sheep.

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