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Identification of Single Nucleotide Polymorphism (SNP) in β-Lactoglobulin Gene and Its Association with Milk Composition in Iranian Indigenous Khalkhali Goats

Research Article

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ABSTRACT

β-lactoglobulin is one of the important milk proteins in several ruminants like goats, sheep and cattle. This study was performed to investigate the genetic polymorphism of exon 7 and 3' UTR region of β-lactoglobulin (*BLG*) gene and its association with milk composition in 120 unrelated individuals of Iranian indigenous Khalkhali goats. Purified polymerase chain reaction (PCR) products (427 bp) were sequenced under standard conditions using Sanger sequencing. Alignment of sequenced fragments against reference sequence leads to identification of one single nucleotide polymorphism (SNP), substitution A (frequency equal to 0.44) to C (frequency equal to 0.56) in the 3' UTR region of *BLG* gene. Observed frequencies of AA, AC and CC genotypes were 0.12, 0.64 and 0.24, respectively. The effects of identified genotypes on milk composition were analysed using general linear model. We found that *BLG* gene genotypes have a significant effect on milk parameters except for lactose percentage ($P<0.05$). The milk of goats with AA and AC genotypes had higher protein and fat percentages, respectively, in compaired with other genotypes ($P<0.05$). Obtained results revealed that, identified genotypes in the *BLG* gene of Khalkhali goats is not in Hardy-Weinberg equilibrium.

KEY WORDS *BLG* gene, genetic variation, Khalkhali breed, milk composition, SNP.

INTRODUCTION

Iran is one of the most likely locations for goat domestication about 10000 years ago (Haenlein, 2007; Naderi *et al.* 2008). Goats are considered an important domestic species in Iran due to regional and cultural resaons. Based on FAOSTAT (2017), Iran with almost 23.2 million goats (eighth in the world), is one of the important locations for goat rearing in the world. Due to different climates and breeding manners, several divers goat breeds have raise up in Iran, including Najdi, Tali, Raini, Markhaz, Bluchi, Mamasani, Adani and Khalkhali. Khalkhali goats are mainly dispersed in khalkhal, Dasht-e-Moghan (Ardabil province) and in some part of Azarbaijan-Sharghi province of Iran. This breed has a small body size (Figure 1) and mostly is breed for meat and milk production. Goat milk in compared with cattle milk, has favorable chemical, physical, organoleptic and nutritional characteristic that makes it a good nutritional source for infants and chidren and also a medicinal food (Silanikove *et al.* 2010).

Most of milk proteins in ruminants are encoded by six genes including four casein genes as cluster of *CSN1S1*, *CSN2*, *CSN1S2* and *CSN3* (Rijnkels, 2002) and also two lactoglobulin genes including α- and β-lactoglobulin (*α-LA*

and *β-LG*) (Martin *et al.* 2002). *BLG* gene contains 7 exons and is located on goat's chromosome 11. This gene encodes a protein with 180 amino acid and mass of 19976 Da in goats (Uniprot accession: P02756) and considered as a important allergens component of milk in cow, sheep and goat (Kapila *et al.* 2013). It is worth to mention that this protein is absent in milk from camels, human and rabbits (Selvaggi *et al.* 2015).

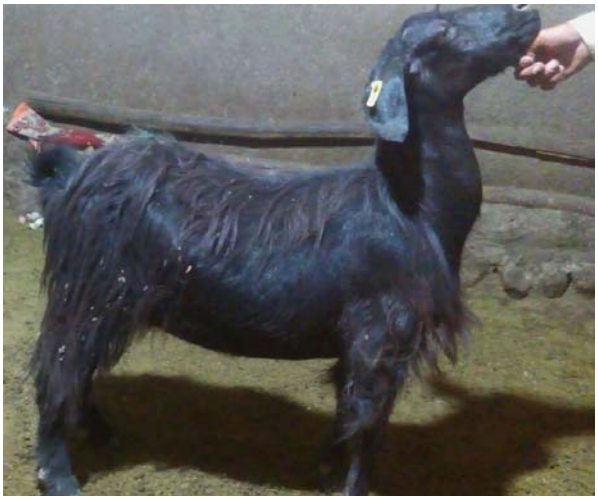

Figure 1 A female Khalkhali goat

Several studies on genetic polymorphisms in the *BLG* gene and their association with the milk-related traits were performed in ruminants such as cattle (Ganai *et al.* 2009; Karimi *et al.* 2009; Vidovic *et al.* 2014; Ozdemir *et al.* 2018) and sheep (Çelik and Özdemir, 2006; Staiger *et al.* 2010; Kawecka and Radko, 2011). Also, previousley studies about milk-related traits in goats (Caravaca *et al.* 2011; Dagnachew *et al.* 2011; Palmeri *et al.* 2014; El Hanafy *et al.* 2015) confirmed the impact of genetic variants on some traites like protein content of milk that is a important attribute for produts like the cheese. An extensive number of genetic diversity have been reported in different parts of *BLG* gene in different species (Sardina *et al.* 2012; Yang *et al.* 2012; Özmen and Kul, 2016). Among them, the polymorphisms at exon 7 define one of important variation effect on milk composition in goat (Kumar *et al.* 2006; El Hanafy *et al.* 2015).

Despite the investigation of genetic variants of *BLG* gene and their effects on milk composition in some Iranian goat breeds (Gharedaghi *et al.* 2016), but no similar information about *BLG* gene variation has been reported in the Khalkhali goat breed.

Therefore the current study was design to investigate about genetic polymorphism of *BLG* gene and its impact on milk composition in Khalkhali goat.

MATERIALS AND METHODS

Samples and DNA extraction

Based on survey information, a total of 120 unrelated individuals were randomly selected from four different herds of Khalkhali goat in the Northwest of Iran according to guidelines of animal care. The sampled animals were completely dependent on the same pastures; they were grazing in the pasture during the day and kept in a roofed place at night. All procedures used in this experiment approved by research council of university of Mohaghegh Ardabili.

Milk records were obtained from all 120 Khalkhali goats, so that, milk of some goats were sampled in the first lactation and others in the second lactation. Individual milk samples were collected manually from morning milking at the end of each month of lactation and were stored at -20 ˚C until further use. For more accuracy, each sample was divided into two fractions for analysis of milk composition including protein, fat, lactose percentage and dry matter content using Milko-Scan FT 6000 (Foss Electric, Hillerød, Denmark).

About 4 mL of blood was collected through the jugular vein of 120 sampled goats using venoject tubes containing lyophilized ethylenediaminetetraacetic acid (EDTA) (EDTA). Total genomic DNA was purified from whole blood using Exgene Cell SV kit (GENEALL Biotechnology co, LTD, Republic of Korea) and the quality of extracted DNA was assessed using 0.8% Agarose gel electrophoresis and Thermo Scientific™ NanoDrop spectrophotometers.

PCR amplification, sequencing, and statistical analysis

A 427 bp fragment of exon 7 and 3' UTR of the caprine *BLG* gene was amplified (Figure 2) using following primers set: Forward 5-CGGGAGCCTTGGCCCCTCTGG-3 and Reverse 5-CCTTTGTCGAGTTTGGGTGT-3 (Kumar *et al.* 2006). Amplification of desired DNA fragment, was carried out in 25 µL volume reaction mixture containing 0.2 mM dNTP, 1.2 mM $MgCl_2$, 1.5U Taq DNA polymerase (Ampliqon), 15 pMol of forward and reverse primer (Invitrogen) and 100 ng genomic DNA. The thermal profile consisted of denaturation at 95 ˚C for 5 min, followed by 33 cycles of 94 ˚C for 30s, annealing at 63 ˚C for 30 s and extension at 72 ˚C for 60 s, with a final extension step at 72 ˚C for 10 min. The PCR products were purfied using Expin™ PCR SV kit from GENEALL company. Then, purified products were sent to Macrogen company (Seoul, South Korea) for sequencing under standard condition using sanger sequencing technology (Sanger *et al.* 1977). Chromas 2.33 (https://technelysium.com.au/wp/chromas/) was used to edit the sequenced fragnments.

Then, the obtained sequences were analyzed and compared using MEGA 6.0 (Tamura *et al.* 2013).

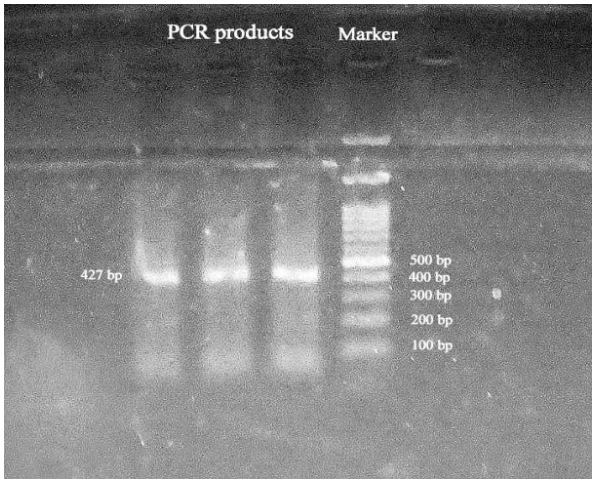

Figure 2 PCR amplification of *BLG* gene (A fragment of 427 bp) in Khalkhali goat

The genotype frequency of single nucleotide polymorphisms (SNPs) was determined by direct counting methods. Finally, a fixed model was used to perform the milk composition association with *BLG* gene polymorphisms using generalize linear model (GLM) procedure of SAS software 9.2 (SAS, 2004). The used model includes herd, SNP genotype and lactation stage as fixed effects:

$$Y_{ijkl=} \mu + H_i + L_j + G_K + e_{ijkl}$$

Where:
Y_{ijkl}: milk traits (Fat, protein, lactose, solid material percentage).
μ: general mean for the certain trait.
H_i: fixed effect of i^{th} herd (i=1, 2, 3 and 4).
L_j: fixed effect of j^{th} lactation (j=1, 2).
G_k: fixed effect of k^{th} genotype (k=AA, AC and CC).
e_{ijkl}: residual effect.

It should be noted that, the significance of deviations was verified with the Tukey-Kramer test. Also, we used chi-square test manually to test the population for Hardy-Weinberg equilibrium:

$$\chi^2 = \Sigma (O_i - E_i)^2 / E_i$$

Where:
O_i and E_i: observed and expected number of individuls for the i^{th} genotype, respectively.

RESULTS AND DISCUSSION

The means for milk composition of Khalkhali goats are present in Table 1. Milk samples were obtained from Khalkhali goats living in natural pastures belonging to Ardabil province during spring and first month of summer. The fat and protein percentage of Khalkhali breed milk in compared with goat breeds like Girgentana (Todaro *et al.* 2005), greek indigenous breed (Kondyli *et al.* 2012), Malaysian local breeds (Jamnapari, Shami and Toggenburg) (Mohsin *et al.* 2019), Italian local goats (Jonica, Mediterranean Red and Garganica), Saanen (Currò *et al.* 2019) and Alpine breeds (Da Costa *et al.* 2014) were low.

Alignment of sequences against reference sequence (Accession number in NCBI: XM_018054689.1) showed a transversion polymorphic site in 3' UTR that leads to substitution of A nucleotide to C nucleotide in position 362 bp (Figure 3), based on the reference sequence, the mutation was placed in 798 position. Based on detected variant, three different genotypes were detected in present study that called AA, AC and CC with frequency 0.12, 0.64 and 0.24 respectively (Table 2).

Statistical analysis revealed that herd as a fixed effect has a significant impact on all investigated milk-related traits. Identified genotypes of *BLG* gene significantly influenced the milk composition parameters, i.e. fat, protein and solid material percent ($P<0.05$). However, observed genotypes had no significant effect on lactose percentage of obtained milk samples. While, milk from goats with AC genotype had significantly higher fat than milk of goats with CC genotypes. Also, AA genotype showed significantly higher protein and solid material in compared with CC genotype (Table 3).

Obtained results based on chi-square test ($P<0.01$) showed that identified genotypes in the *BLG* gene of Khalkhali goats is not in Hardy-Weinberg equilibrium (Table 2). As mentioned earlier, the milk of goats with AC genotype has higher fat percentage and proper composition, therefore, it seems that this genotype may be a convenient option for breeders and ranchers. The results also revealed that the selection was probably against CC genotype due to lower fat and protein percentage in its milk in compared with the other two genotypes. The results are consistent with this fact that the percentage of fat and protein in goat's milk is considered a key factor among ranchers of area, in order to produce more butter and cheese.

Kumar *et al.* (2006), amplified the *BLG* gene from exon 7 to the 3' flanking region (426 bp) as we did, and genotyping of this region of gene revealed the presence of two alleles and three genotypes.

Table 1 Means of milk composition traits (Means±Standard deviation) in Khalkhali goat

Traits	Herd 1	Herd 2	Herd 3	Herd 4
Individuals	44	31	26	19
Fat %	2.95±0.28	2.81±0.26	3.16±0.48	2.9±0.34
Protein %	3.19±0.35	3.29±0.49	3.35±0.45	3.43±0.22
Lactose %	4.73±0.38	4.81±0.38	4.79±0.53	4.87±0.30
Solid material(without fat) %	8.81±0.69	8.89±0.82	8.87±0.51	8.92±0.48

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

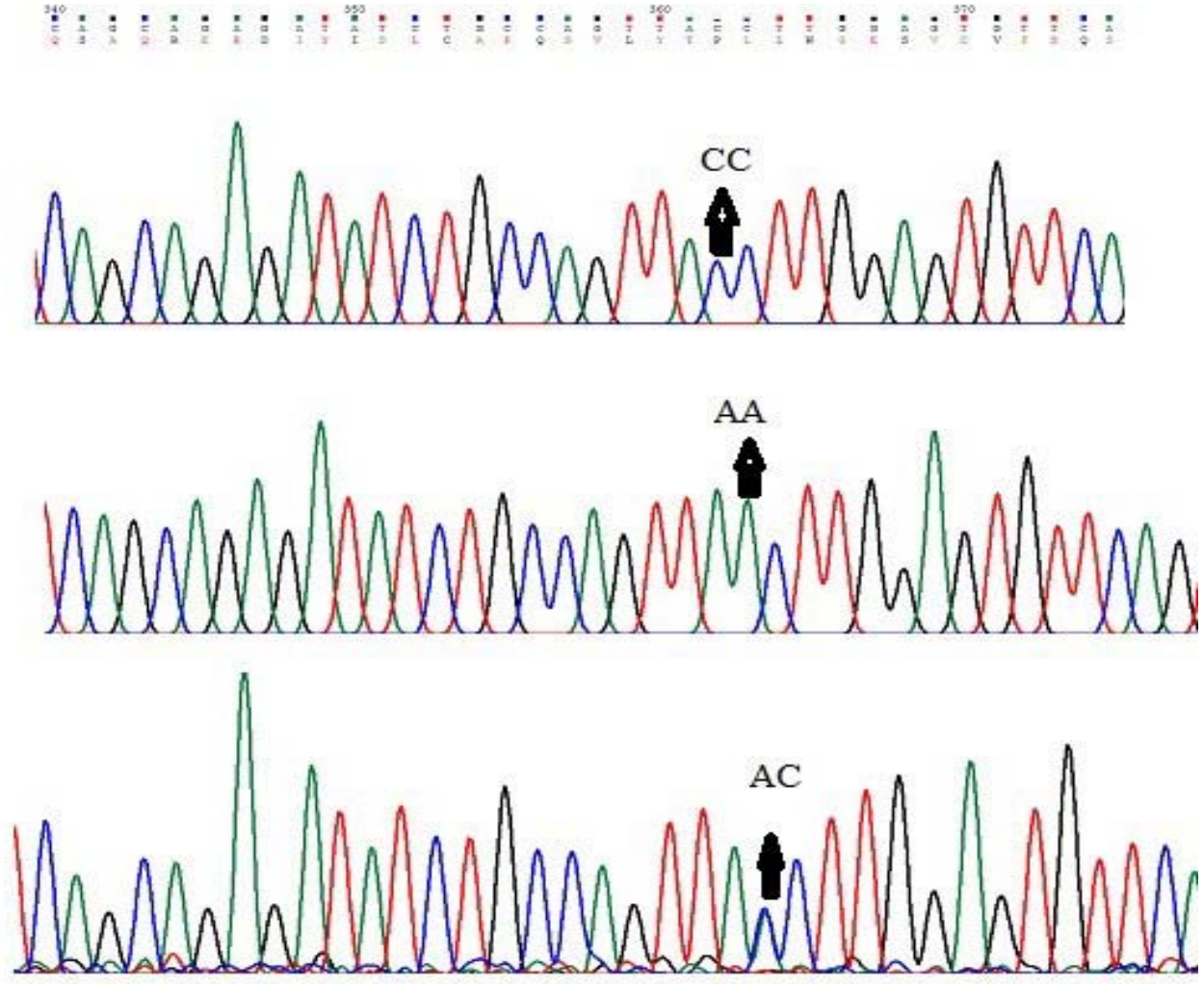

Figure 3 DNA sequencing chromatograms of CC, AA and AC genotypes of *BLG* gene in Khalkhali goat

Table 2 Allelic and genotypic frequencies of the *BLG* gene (n=120) in Khalkhali goats

Allele/genotype	No. of individual		Observed frequency	Expected frequency under HWE
Allele	-	A	0.44	-
	-	C	0.56	-
Genotype	14	AA	0.12	0.19
	77	AC	0.64	0.49
	29	CC	0.24	0.32

HWE: Hardy-Weinberg equilibrium.

Table 3 Results of association analysis of BLG genotypes with milk composition in Khalkhali goat breed

Traits	Genotypes (Least squares mean± Standard error)			P-value
	AA	AC	CC	
No. of individual	14	77	29	
Fat %	2.92±0.32^{ab}	3.61±0.27^{a}	2.67±0.57^{b}	0.032
Protein %	3.42±0.21^{a}	3.27±0.17^{ab}	3.05±0.15^{b}	0.045
Lactose %	4.71±0.26^{a}	4.74±0.32^{a}	4.48±0.37^{a}	0.158
Solid material %	8.96±0.23^{a}	8.61±0.27^{ab}	8.23±0.14^{b}	0.038

The means within the same row with at least one common letter, do not have significant difference (P>0.05).

In addition to mentioned study, identification of variants in this region in our work and other studies (Pena *et al.* 2000; El Hanafy *et al.* 2015) revealed that this part of *BLG* gene considered as a variable region which affects milk composition.

Although, variation detected in this part of caprine *BLG* gene in present study, dose not generate any changes in the amino acids, but it may effect on the regulation of gene or mRNA splicing or linked to other polymorphisms in the coding area, which may have effect on gene expression level.

Evidence suggests that variants in nonexonic regions can have major effects on the phenotypes (Van Laere *et al.* 2003). In accordance with the current study, the results of investigation in sheep, goat and cattle breeds have shown that *BLG* polymorphisms significantly effects on milk composition (Dettori *et al.* 2015; Selvaggi *et al.* 2015; Cardona *et al.* 2016; Gras *et al.* 2016). However, there are some studies that failed to detect significant effect of variants on milk composition (Karimi *et al.* 2009).

CONCLUSION

This study has been investigated the association between the BLG genotypes and milk composition traits in Khalkhali goat breed. The obtained results indicate the relationship between identified genotypes and fat, protein percentage and solid content of Khalkhali goats milk. We found that the milk of goats with BLG AC genotype had higher fat and lactose percentage and also proper protein and solid content. Based on obtained results, we suggest that AC genotype is the best option for selection, as the breeders.

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