

Article

Effect of a Novel Variant with Let-7c MicroRNA Gene on Litter Size in Markhoz Goats

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Abstract: This study was focused on identifying the effects of single nucleotide polymorphisms (SNPs) located on an entire region of the let-7c miRNA gene with consideration of its ability to promote litter size in Markhoz goats. The Markhoz goat, the native breed in Iran, is important for its reproductive traits, such as litter size. DNA polymorphism of let-7c miRNA gene was revealed and considered for further studies for its effect on litter size in Markhoz goats. PCR-SSCP analysis investigated different band patterns for this miRNA; however, sequencing results have detected only an A to T substitution located five nucleotides downstream of the let-7c miRNA gene. The chi-squared test showed that the let-7c miRNA gene locus was out of the Hardy–Weinberg equilibrium (HWE) and has significant effect ($p < 0.05$). Furthermore, the least-square analysis indicated that the let-7c miRNA gene does not affect prolificacy in the Markhoz goat ($p > 0.05$). In sum, all loci failed to have a significant effect on the litter size trait ($p > 0.05$). Moreover, years of kidding and parity had no significant impact on let-7c_S ($p > 0.05$); however, the let-7c_B affected the litter size trait significantly ($p < 0.05$). Additionally, binary logistic regression and chi-square analysis revealed that allele A of the detected SNP within 3' UTR region of the let-7c gene had a non-significant effect on litter size in the studied goats ($p > 0.05$).

Keywords: litter size; let-7; microRNA; 3' UTR; sequencing



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1. Introduction

Litter size is one of the most important reproductive traits in multiparous animals; thus, prolificacy is economically crucial in small ruminants such as goats. In addition, the ovulation rate is essentially connected to the kidding rate. The heritability of reproductive traits is low; thus, using marker-assisted selection in combination with traditional methods can improve these traits in animals [1]. Moreover, it is necessary to identify candidate genes that play an important role in critical traits in animals, especially litter size traits [2].

MicroRNAs are small non-coding single-stranded RNA molecules (about 22 nucleotides long) that account for 2–5% of mammalian genes and also show an important regulatory role in up to 60% of functional genes [3]. Approximately half of the known miRNAs are located in intragenic regions. The other half are located in genes with about 40% and 10% in introns and exons, respectively [4]. In general, they will be co-expressed with their hundreds of mRNA targets [4,5]. They are involved in different biological processes, including cell proliferation, differentiation, apoptosis, tumorigenesis, hormone secretion, and metabolism [6]. New studies recommend that single-nucleotide polymorphisms (SNPs) occurring in miRNA coding genes or their binding sites on target genes can alter functions of miRNA and/or their target genes [7], resulting in increased or decreased expression of that gene.

A recent study by [8] showed that there is a significant relationship between the miR-9 gene and litter size in Markhoz goats. Another study conducted on porcine prolificacy reported that specific miRNAs, such as miR-224, miR-99a, let-7c, miR-181c, miR-214,

and miR-21, may influence pig fertility. Additionally, the important role of miRNAs in regulating and increasing ovulation rate was clarified [9].

Lin-4 and let-7 are the first two known miRNAs that were discovered in *Caenorhabditis elegans* and regulate stem-cell division and differentiation timing [10]. Let-7 family miRNAs are conserved across invertebrates and vertebrates and consist of 11 closely related genes [11]. It is reported that let-7c has a key regulatory role in follicle-stimulating hormone secretion in mouse follicle cells [12].

Considering the importance of SNPs in altering functions of miRNAs and their target genes, and considering that miRNAs may affect animal performance throughout gene regulation the aim of the present study was to identify possible variants within the let-7 miRNA gene as well as its target genes and to investigate its effects on litter size at birth in Markhoz goats.

2. Materials and Methods

All procedures used during the present study were approved by the Animal Care Committee of University of Kurdistan, Sanandaj, Iran (protocol number: IR.UOK.REC.1401.017; Approval date: 5 November 2022). Blood samples of Markhoz goats were obtained from the Markhoz Goat Performance Testing Station in Sanandaj, Kurdistan, Iran, during the breeding season. Ages of animals ranged from 3 to 5 years, with a history of multiple births (twin or triplet births) and single births, respectively. Samples were collected from 88 female and 32 male goats along with their litter size records for investigating possible single nucleotide polymorphism of studied genes and their associations with litter size. The Markhoz goat population is an endangered goat population, of which only 340 remain, including 156 adults (116 females and 40 males) and 184 kids under one year of age. However, only 88 does were available, and we used all females in the herd with a history of parturition. All goats were maintained in the same conditions of feeding and rearing. Venous blood (5–6 mL) was collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and immediately delivered to the lab in an ice box.

2.1. DNA Isolation and PCR

DNA samples were extracted from blood samples according to the salting out method [13]. After checking the quality and quantity, DNA was diluted to a final concentration of 50 ng/ μ L in deionized water and stored at -20°C for further use. The OligoAnalyzer online tool was used to design specific primers based on the GenBank sequence (NCBI Accession No. NC_030808.1) for the let-7c miRNA gene. The sequence of forward and reverse primers was “AGTCCTTAGGTGTATGGCTG” and “CACAGAAATTGGCTCAATCA”, respectively, with an annealing temperature of 57°C , producing a 225 bp segment. A total volume of 20 μ L reaction mixture, which contained 10 μ L Master Mix, 1 μ L Primer, 2 μ L DNA, and 7 μ L H_2O , was used for amplification DNA. The PCR program was performed using a Palm-cycler PCR machine (Corbett CG1-96) at 57°C for the let-7 miRNA gene. PCR products were treated by electrophoresis on 1.5% agarose gel at 80 V for 30 min and imaged on an UV Transilluminator unit (Biostep UXFT, Saxony, Germany).

2.2. SSCP Assay

SSCP is a cost-effective method for detecting mutations and has an appropriate sensitivity if fragments from 150 to 300 bp in length are investigated. The PCR products (5 μ L) were mixed with 5 μ L SSCP buffer (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole, and 0.025% bromophenol blue), and heated at 98°C for 10 min, and then chilled on ice for 7 min. The denatured DNA samples (2 μ L) were then subjected to 12% PAGE (polyacrylamide gel electrophoresis) in $1 \times$ TBE (Tris-borate EDTA) buffer. The prepared acrylamide gel was electrophoresed under 140 V for 15 h at 4°C . Subsequently, the gel (29:1, acrylamide:bisacrylamide) was stained with 0.1% silver nitrate [14]. Ultimately, the polymorphisms were detected and the PCR products of the different electrophoresis pat-

terns were selected for sequencing. The sequences were visualized using FinchTV software v. 1.4 and blasted using the NCBI online tool (Figure 1).

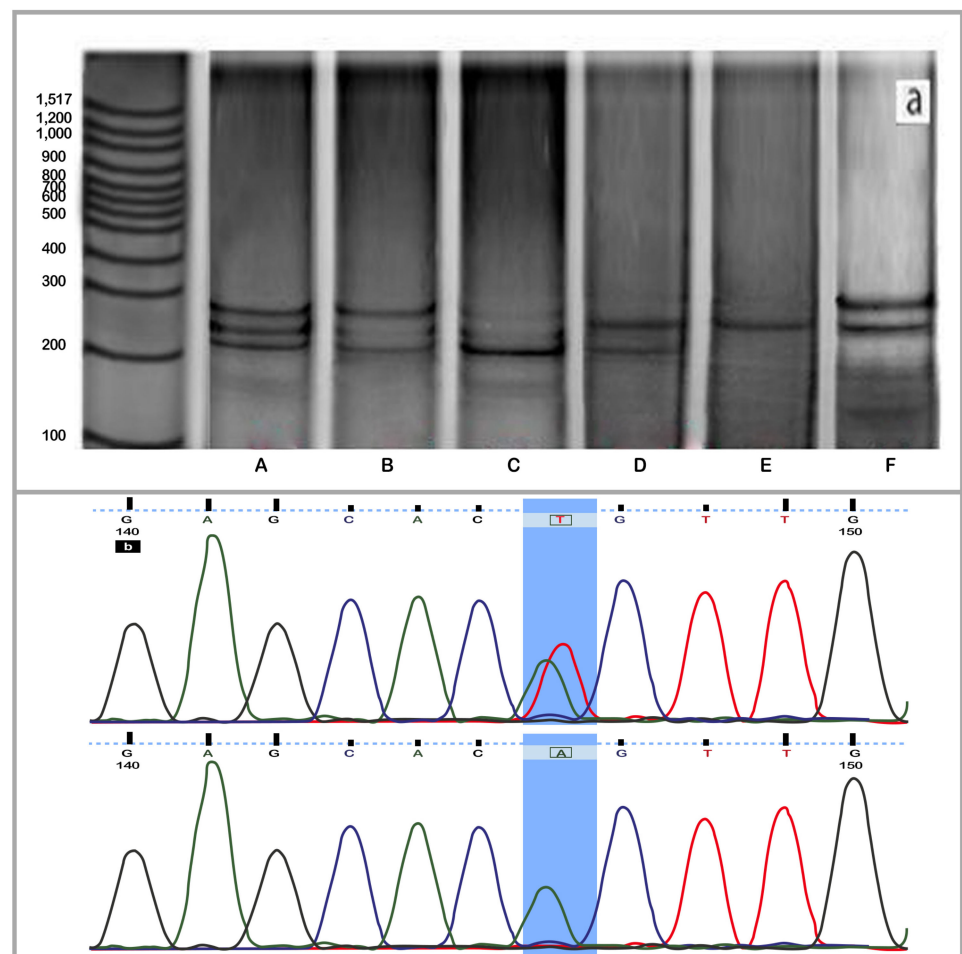


Figure 1. SSCP patterns for 3' UTR of (a) the let-7 miRNA gene (225 bp), and the sequence chromatogram (b) for the let-7 miRNA gene. The chromatogram in the top represents the sequence having SNP, and the chromatogram in the bottom represents the sequence without SNP. A, B, C, D, E and F are different patterns of let-7 miRNA gene polymorphism.

2.3. Statistical Analysis

Data for the litter size of 88 goats were used in the present study. Least squares analysis was used for investigating the effects of different SSCP patterns/SNPs on litter size. Furthermore, the logistic model was applied to detect the effects of a single allele on litter size. The following statistical model was used to fit data for comparing difference of litter size between genotypes and alleles:

$$y_{ijlmo} = \mu + G_i + A_j + K_l + F_m + e_{ijlmo} \quad (1)$$

where y_{ijlmo} is the phenotypic value of litter size; μ is the population mean; G_i is the fixed effect of the genotype (in least-square analysis) or allele (in logistic regression analysis); A_j is the fixed effect of the age of the animal; K_l is the fixed effect of the kidding year; F_m is the effect of inbreeding of each animal (derived from pedigree) as a covariate; and e_{ijlmo} is random error. Estimations were achieved using the general linear model procedure (least-square and logistic models) of SAS v. 8.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. PCR-SSCP Analysis of the let-7 MiRNA Gene in Markhoz Goats

In the present study, we have used the method introduced by [15–17] for the naming of SSCP patterns. In SSCP analysis, electrophoresis of the let-7 miRNA gene revealed six band patterns, including A, B, C, D, E, and F (Figure 1).

3.2. DNA Sequence Analysis

In the let-7c miRNA gene, amplified fragments with different PCR-SSCP patterns were sequenced. After sequencing, the comparison between the sequenced data and the GenBank reference sequence of let-7c (NC_030808.1) was performed. An A to T substitution located five nucleotides downstream of the let-7c miRNA gene was identified.

3.3. Hardy–Weinberg Equilibrium

Detailed information of sample size (32 males and 88 females), genotypic and allelic frequencies, HWE p -value, polymorphic information content (PIC), and heterozygosity (He) of detected SNP in the Markhoz goat population is presented in Table 1. The chi-squared test showed that the let-7c miRNA gene locus was not in HWE ($p < 0.05$). The PIC value of the let-7c miRNA gene was 0.278, representing a low genetic diversity regarding discovered SNP in the studied population. The observed heterozygosity was 0.042 in let-7c miRNA gene.

Table 1. Results of Markhoz goat population genetic analysis for identified locus.

Loci	Sample Size	Genotype	Genotype Frequency	Allelic Frequency	HWE	PIC	He
let-7c	120	AA	23	A 0.212	$p = 0.00$	0.278	0.042
		AT	5	T 0.788			
		TT	92				

HWE: Hardy–Weinberg equilibrium; PIC: polymorphism information content; He: heterozygosity.

3.4. Association Analysis of Polymorphisms of let-7 MiRNA Gene with Litter Size

The least-square analysis for the litter size of five identified genotypes showed no significant effect between patterns of the let-7c miRNA gene ($p > 0.05$). In let-7c_S, the year of kidding and age of animal have not shown any significant effect ($p > 0.05$). However, in let-7c_B analysis, the litter size was significantly affected by year of kidding and age of animal ($p < 0.05$). The LS-Means of all genotypes of each gene are contrasted with each other according to reported p -value in Table 2.

Table 2. Least square means and standard errors for litter size of different genotypes of detected SNPs in Markhoz goats.

Locus	p -Value	Genotype	Sample Size (No. of Goats)	LS-Mean \pm SE
let-7c_B	0.283	A	108 (38)	1.456 \pm 0.099
		B	27 (8)	1.339 \pm 0.106
		C	11 (3)	1.543 \pm 0.156
		D	35 (13)	1.329 \pm 0.119
		E	48 (17)	1.509 \pm 0.109
		F	7 (2)	1.335 \pm 0.185
let-7c_S	0.363	AA	48 (17)	1.494 \pm 0.107
		AT	7 (2)	1.319 \pm 0.184
		TT	181 (62)	1.405 \pm 0.091

let-7c_B: Results for let-7 miRNA gene based on SSCP band patterns; let-7c_S: Results for let-7 miRNA gene based on sequencing.

Because goats can have multiple genotype patterns, the sample size and number of goats were different.

For studying the effects of each allele on litter size, the data were analyzed, and results are presented in Table 3.

Table 3. The parameters of logistic analysis of identified SNP allele effects in Markhoz goats.

Loci	Allele	Low LS *		High LS		P_{χ^2}	P_{logistic}
		0	1	0	1		
let-7c	A (0) T (1)	13	52	5	13	0.471	0.478

*LS: Litter size.

We used binary logistic regression for investigating the effects of a single pattern/allele of detected SNPs (Table 4). The result revealed that allele A of the detected SNP on the downstream region of the let-7c miRNA gene did not significantly affect the litter size of goats ($p > 0.05$), and chi-square analysis confirmed it at a level of 0.05. The results failed to show significant effects of all identified patterns in the let-7c miRNA gene ($p > 0.05$).

Table 4. Results of logistic analysis of identified SNP allele effects in Markhoz goats.

Locus	Allele	p -Value
let-7c_B	A	0.5812
	B	0.6825
	C	0.6201
	D	0.2496
	E	0.4788
	F	0.9758
let-7c_S	A	0.9714
	T	0.9012

let-7_B: Results for let-7 miRNA gene based on SSCP band patterns; let-7_S: Results for let-7 miRNA gene based on sequencing.

4. Discussion

To the best of our knowledge, this is the first study that investigated the effects of possible variants within 3' UTR region of the let-7c miRNA gene on litter size in Markhoz goats. Because of the small population size of the Markhoz goat, we had to use a small sample size of only 88 adult females for this research. This valuable goat breed has been maintained in the Agricultural Organization of Kurdistan, and their reproductive information was regularly recorded there. For this reason, the sampling situation was difficult, and only 88 goats with 222 records of kidding were available for conducting this study.

In the goat genome, the let-7c miRNA gene is located on chromosome 1. Mammalian ovaries play a key role in the performance of reproduction traits. For example, two miRNAs that may affect litter size regulation are mir-181 and let-7c [9].

Research on characterization and expression analysis of microRNAs in sheep ovaries indicated that specific members of let-7 influence mammalian reproduction, development, cell proliferation, and apoptosis [18].

Another research [19] reported that let-7 miRNA is important for embryo implantation. In addition, work on proliferation of human cells showed that let-7 miRNA is one of the master regulators of the cell proliferation pathway [11]. Results of identification of miRNAs associated with the follicular–luteal transition in the ruminant ovary has been shown; this miRNA family is critical for angiogenesis in the developing process [20]. Specifically, let-7c miRNA can regulate follicle stimulating hormone secretion in mouse follicle cells and follicular development [6,9].

The sequencing of PCR products of all different patterns revealed only an A to T substitution located five nucleotides downstream of the let-7c miRNA gene. The least-square analysis for this variant detected no significant effect on litter size in Markhoz goats.

In let-7c_B, six different genotypes at an identified mutated locus revealed no significant difference at the level of 0.05. Other detected patterns on studied genes in this research failed to have a significant effect on litter size. One possible reason behind the non-significant effect of identified polymorphisms could be the related animals used for the study caused by the small size of the population. According to previous studies, two of five effective ovine miRNAs contributed at four stages of follicular and luteal development [17]. These results led to important information that the let-7c is one of the most influential miRNA on litter size in goats.

5. Conclusions

For investigating the effect of a single allele on litter size, we categorized the data in two low and high litter sizes based on the obtained mean: low if mean of litter size was less than 1.5 and high if mean of litter size was greater than 1.5. Subsequently, the data were used to fit logistic regression analysis to investigate the effect of each allele on litter size. The results of logistic regression confirmed that pattern A of the let-7c miRNA gene did not significantly affect litter size in Markhoz goats.

On the other hand, using binary logistic regression for investigating the effect of each allele on litter size, an A to T substitution was detected in sequencing analysis within 3' UTR region of the let-7c miRNA gene; however, this study did not reveal a significant effect of this SNP on litter size in Markhoz goats.

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