

Association between Melatonin Receptor 1A (*MTNR1A*) Gene Polymorphism at the *MnII* Site and Production Traits in Shal and Crossbreeding between Shal and Romanov

Research Article

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ABSTRACT

The objective of this study was to evaluate the relationship between polymorphism at the *MnII* site of *MTNR1A* gene and production traits of Shal and Romanov crossbreeding. The crossbreeding between Shal and Romanov was done on 600 Shal ewes. For this reason, Shal ewes were laparoscopically and artificially inseminated with the semen of Romanov breed. For the preparation of genomic DNA, a total of 90 ewes, either Shal (n=50) or Shal × Romanov (n=40), were used and an 824 bp fragment of exon 2 of the *MTNR1A* gene was amplified. In this research, two genotypes MM and Mm were detected in the study Shal population with frequencies of 0.78 and 0.22, respectively, while MM and mm genotypes of the study crossbreeds were detected with 0.125 and 0.0875 frequencies, respectively. However, the mm and Mm genotypes were not detected in Shal and Shal × Romanov crossbreeds, respectively. The *MnII* marker site in Shal × Romanov breeds had a significant effect ($P>0.05$) on body weight after 3 months (BW3) as the ewes with MM genotypes had significantly higher BW3 than mm individuals. However, no significant difference was observed between MM and mm marker sites for birth weight (BW) and 6-month body weight (BW6). The same results were observed for Shal breeds as when MM and Mm genotypes were detected for BW, BW3 and BW6 traits. In conclusion, in this study, mm genotype as a measurement for litter size in sheep breeds was successfully introduced into Shal × Romanov crossbreeds, while their production traits were not affected by *MnII* genotypes.

KEY WORDS crossbreed, *MTNR1A*, Romanov, Shal.

INTRODUCTION

Remarkable seasonal variations are observed in the reproductive activities of small ruminants living in temperate latitudes (Luridiana *et al.* 2015). Thus, a great alteration is induced by lamb reproductive seasonality and its consequent meat market price. Alternatively, out-of-season reproduction is induced through a wide use of hormonal treatments in some countries. However, there is a need for a search for some other methods due to the increasing de-

mand for free-hormone products (Martinez-Royo *et al.* 2012). Nonetheless, identification of informative genetic markers has been suggested to improve selection efficiency for reducing breeding seasonality in sheep (Moradi *et al.* 2014). In a response to photoperiodism, animals are engaged in the secretion of differential melatonin to regulate their reproductive activities (Saxena *et al.* 2014). The light signals relayed to the pineal gland by several neurons from the retina, known as photoperiodic information, are translated into the daily cycle of melatonin secretion, the level of

which is high at night and low during day time (Shahroudi *et al.* 2006). Accordingly, the secretion of gonadotrophin-releasing hormone (GnRH) is controlled by the seasonal rhythms of melatonin secretion that serves as a seasonal messenger in endocrine tissues at the hypothalamic level (Luridiana *et al.* 2014). The alternating presence or absence of female ovulation and male sperm production is dependent on luteinizing hormone secretion, the levels of which are induced by the corresponding changes in GnRH release (Shahroudi *et al.* 2006).

Melatonin reproductive effects occur in the premammillary hypothalamus (Luridiana *et al.* 2015) and the hypothalamic suprachiasmatic nucleus is the site of the circadian clock where melatonin circadian effects are mediated by the pharmacologically specific G-protein-coupled melatonin receptors with high affinity (Jia *et al.* 2012), through which melatonin exerts its effects when binding to them. However, it seems that the reproductive activity is only regulated by MT1 from among the receptors involved (Luridiana *et al.* 2015).

In an investigation for sheep sexual activity, Notter *et al.* (2003) showed that the genotype of this gene might become as a particular marker based on the association between different allelic forms at MT1 locus and sheep reproductive activity.

Reppert *et al.* (1994) were the first to clone melatonin receptor 1A gene (*MTNR1A*) as a high-affinity subtype in mammals. It should be noted that from among any other melatonin receptor subtypes like *MTNR1B* and *MTNR1C*, only *MTNR1A* appears to be engaged in the seasonal reproductive activity regulation (Saxena *et al.* 2014; Trecherel *et al.* 2010). An association between *MTNR1A* gene and seasonal reproductive activity has been evidenced by several studies on different animal species (Luridiana *et al.* 2014).

Circadian variations in melatonin concentrations have been repeatedly proposed to be caused by *MTNR1A* as a candidate gene via photoperiodic control of seasonality (Mura *et al.* 2014). *MTNR1A* gene, consisting of two exons divided by a large intron, has been mapped in the ovine chromosome 26. The exon one shows low degree of polymorphism. It has been shown that exon 2 of *MTNR1A* gene, which is involved in the coding process of ovine MT1 receptors is highly polymorphic, while changes in the second exon would make the differences in the structures of the receptors. The 2 restriction fragment length polymorphism (RFLP) sites for *MnII* and *RsaI* enzymes are derived from exon 2 of the gene encoding MT1 receptor in sheep. The characteristic pattern of digestion by this enzyme is characterized by a mutation at *MTNR1A/MnII* site, which leads to the absence (–or M) of *MnII* specific cleavage site at position 605 of the coding sequence (Hatami *et al.* 2014). The 3 genotypes of “M/M”, “M/m” and “m/m”, which were pre-

viously described as +/+, +/- and -/- (Trecherel *et al.* 2010), are discriminated by the presence or absence of this mutation.

Sheep, goat and buffalo were selected to carry out the studies of relationship between this gene polymorphism and reproductive activity. In this regard, a reproductive association with the polymorphic sites identified in *MTNR1A* gene was shown by some sheep breeds like Merinos d'Arles, small tailed Han sheep, Awasi and Sarda (Saxena *et al.* 2014).

A local sheep breed is Shal, which constitutes a population of more than 600000 heads in Iran. The breed is large-sized, fat-tailed, and predominantly black or brown with white spots on the front head, while being well-adapted to harsh climates. It is mainly raised for its meat as the most important protein source in Iran. Following a random exposure of Ewes, which usually lamb 3 times every 2 years, to 18-month rams, lambing occurred within one season, i.e. from mid-January to mid-March (Hossein-Zadeh, 2015).

One of the breeds renowned for its long breeding season, early sexual maturity and high prolificacy is Romanov. However, poor carcass quality and relatively low growth rate are of the typical features of this breed in comparison to the traditional meat species. Application of a commercial crossing approach with the meat-type breeds is a simplest and fastest way of improving growth and carcass quality in Romanov lambs (Kuchtik *et al.* 2012). Crossbreeding between Romanov and Shal were used to improvement of prolificacy in Shal breed. In the current study, we considered the polymorphism of exon 2 of *MTNR1A* at *MnII* site as a marker for prolificacy and investigated its relationship with the production traits of Shal and Romanov crossbreeding.

MATERIALS AND METHODS

Experimental animals and their management:

This experiment was carried out at a sheep farm in Ismaeel Abad (longitude: 49.66° E and latitude: 36.46° N) located in Qazvin province, Iran. Shal and Romanov crossbreeding were done on 600 Shal ewes. To this purpose, Shal ewes (1-2 years old) were laparoscopically and artificially inseminated with the semen of Romanov breed. An integration of natural pasture and concentrate feed under natural photoperiod was followed the ewes since birth. All the lambs were born indoors and a similar nutrition status was considered for all sampled individuals within each breed.

Preparation, amplification, and digestion of genomic DNA

A total of 90 ewes, either Shal (n=50) or Shal × Romanov (n=40), were used. Five mL of blood was collected from

the jugular vein in ethylenediaminetetraacetic acid (EDTA)-coated tubes. Ovine genomic DNA was extracted using phenol-chloroform method as previously described by Psifidi *et al.* (2015) and maintained at -20 °C until use. Approximately, 200 ng of genomic DNA was subjected to polymerase chain reaction (PCR) by using specific primers synthesized by Sina colon, Iran. The primers were those used by Messer *et al.* (1997) (sense primer 5'-TGTGTTTGTGGTGAGCCTGG-3' and antisense primer: 5'-ATGGAGAGGGTTTGCCTTA-3') from the sequence of Exon II of the ovine *MTNR1A* gene (GeneBank U14109). Polymerase chain reaction (PCR) was carried out in a volume of 25 µl containing 200 ng of DNA, 1XPCR buffer, 2.0 mmol of MgCl₂, 0.2 mmol of each dNTP, 10 pmol of each primer, and 2 U of TaqDNA polymerase (Fermentas, Canada). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 repeated cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min on a thermal cycler (Bio-Rad, T100). The PCR products were resolved by electrophoresis on 2% agarose gel in parallel with 250 bp of DNA marker ladder (Fermentas). Then, they were separated by electrophoresis on 2% agarose gel. After amplification, 7 µL of the PCR product was digested with 2 units of *MnlI* endonuclease at 37 °C for 4 hours following a deactivation process at 65 °C for 20 minutes.

Statistical analysis

For the genotyping of the study samples, the digested fragments were electrophoresed on 3% agarose gel and stained with ethidium bromide. Allelic frequencies were determined by direct counting of the observed genotypes. The χ^2 test was used to determine Hardy-Weinberg (HW) equilibrium of the mutation by using following equation (GenAllex Software).

$$\chi^2 = \sum_{i=1}^m \sum_{j=1}^i \left[\frac{(O_{ij} - E_{ij})^2}{E_{ij}} \right]$$

The significance of differences between the treatments was evaluated using the t-test. The results were expressed as mean ± SEM and the statistical significance was accepted at $P < 0.05$. The data were analyzed using a statistical software (SPSS, 2011).

RESULTS AND DISCUSSION

PCR-RFLP analysis of exon 2 of *MTNR1A* gene

In current study 824 bp fragment of exon 2 of Shal and Shal × Romanov *MTNR1A* gene were analyzed by RFLP (Figure 1).

The presence and absence of 303 bp fragment were referred to as alleles m and M, resulting in 236 and 67 bp products. Thus, the 3 genotypes of MM, Mm, and mm were detected based on 236, 236 and 303, and 303 fragments, respectively. In our study, the 2 genotypes of MM and Mm were detected in the study population of Shal with the frequencies of 0.78 and 0.22, respectively (Figure 2 and Table 1). However, MM and mm genotypes were detected in the crossbreeds under study with 0.125 and 0.0875 frequencies, respectively. Mm and mm genotypes were not detected in Shal × Romanov and Shal breeds, respectively (Figure 3 and Table 1). MM and Mm frequencies were significantly higher in Shal compared to Shal × Romanov crossbreeds, and that of mm was significantly higher in the study crossbreeds ($P < 0.05$). The allelic frequencies of 0.89 and 0.125 were observed for M allele and 0.875 and 0.11 for m allele at *MTNR1A* locus in Shal and Shal × Romanov crossbreeds, respectively (Table 1).

Contrary to our results, Chu *et al.* (2006) demonstrated an association between MM genotype and non-seasonal estrus in ewes, as well as a relationship between mm genotype and seasonal estrus in ewes when investigating non-seasonal estrous breeds (Small Tail Han and Hu ewes) and seasonal estrous breeds (Dorset, Suffolk, and German Merino ewes).

Many studies indicated an association between the homozygous genotype for the absence of a polymorphic *MnlI* site at position 605 of exon 2 of *MTNR1A* gene and seasonal anovulation in ewes (Chu *et al.* 2006). In our research, the χ^2 test confirmed that only the study Shal sheep population was in HW equilibrium. Perhaps, this was due to the limited number of crossbred population and non-random mating.

Nevertheless, congruent with our results, Carcangiu *et al.* (2009) reported that the ewes of MM genotypes lambled in autumn following an oestrus in spring. Having a very high frequency (0.81%) among their study ewes, M allele surely played a very important role in this process. Though Notter *et al.* (2003) demonstrated that the various sheep breeds reared in North America were sufficiently influenced by a single M allele leading to a reproductive seasonality, Pelletier *et al.* (2000) found that MM homozygous ewes of Merino d'Arles sheep breed showed oestrus in spring. In their study on native Iranian breeds of sheep, Ghiasi *et al.* (2006) demonstrated that the sheep, which genetically had a potential to show estrus during short and long days, indicated 2 genotypes at *MTNR1A* locus with the frequencies of 0.7 (MM) and 0.3 (Mm) in the Karakul breed, and 0.58 (MM) and 0.42 (Mm) in the Shal breed. Also, in another study on native Iranian breeds, i.e. Zel and Naeini, Moradi *et al.* (2014) reported that M allele was predominant in either Zel or Naeini lambs.



Figure 1 Electrophoresis pattern of PCR product of exon 2 of *MTNR1A* gene (824 bp) in Shal and Shal × Romanov sheep (2% agarose gel) Lanes 1-3 and 4-5, PCR amplification product for Shal and Shal × Romanov, respectively, and M: DNA molecular marker (250 bp)

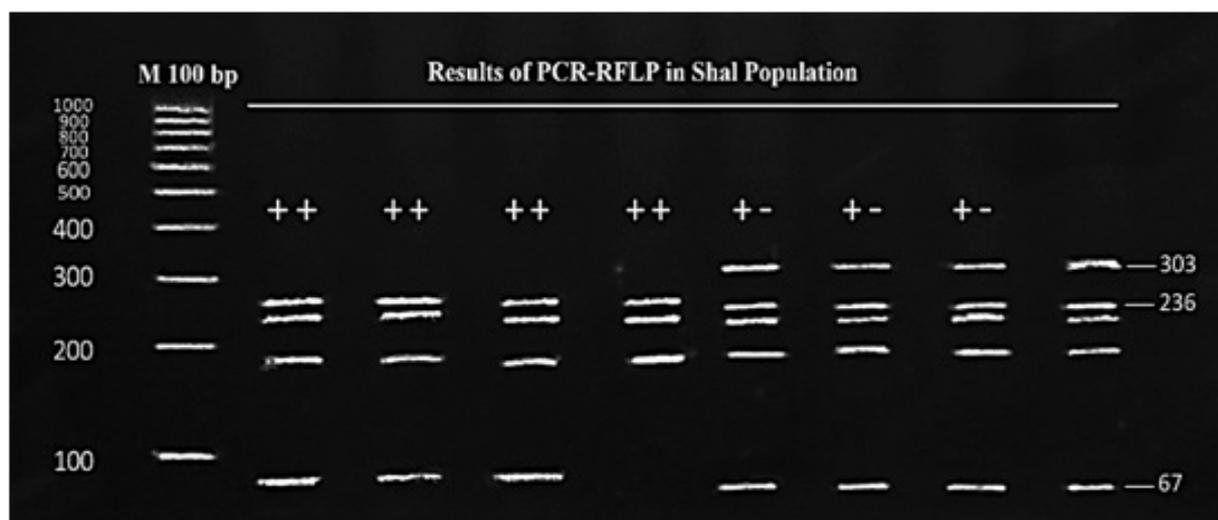


Figure 2 RFLP Genotyping of *MTNR1A* gene by *MnlI* in Shal breed

Table 1 Allele and genotype frequencies of exon 2 of *MTNR1A* gene for *MnlI* site in Shal and Shal × Romanov breeds

Breed	Allele frequency		Genotype frequency		
	M	m	MM	Mm	mm
Shal	0.89	0.11	0.78	0.22	0
Shal × Romanov	0.125	0.875	0.125	0	0.875
P-value	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001

The genotypic frequencies for MM, Mm, and mm genotypes were 0.52, 0.25 and 0.23 in Zel breed and 0.60, 0.22 and 0.18 in Naeni breed. Furthermore, they showed that MM genotype had the lowest mean for litter size significantly. In general, the highest mean measurements for litter size were obtained from mm followed by Mm, whereas the minimum mean measurements were achieved from MM in both assessed breeds.

Even in Sarda sheep breed exhibiting an anoestrous period in late-winter/spring, polymorphisms within *MTNR1A* gene lead to some advances in reproductive activity resumption (Carcangiu *et al.* 2009).

Additionally, Luridiana *et al.* (2015) evidenced an relationship between *MTNR1A* gene polymorphism and a resumption of reproductive activity in adult Sarda sheep breed in spring.

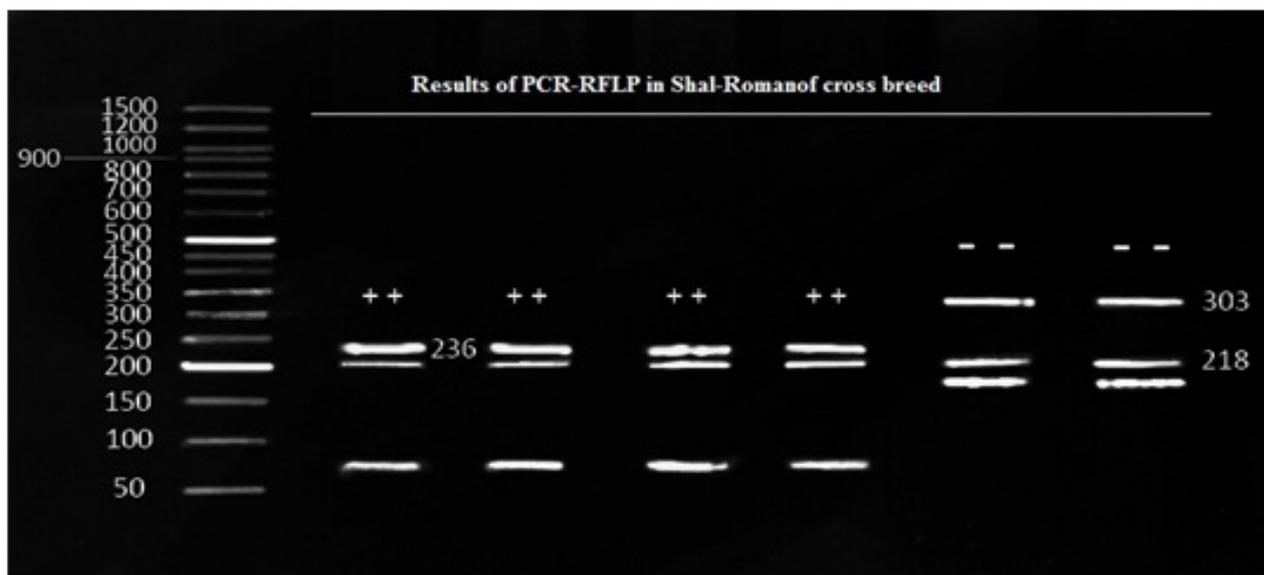


Figure 3 RFLP Genotyping of *MTNRIA* gene by *MnlI* in Shal × Romanov breed

However, no association between *MNTRIA* polymorphisms and seasonality of reproduction in Ile-de-France ewes was found by Hernandez *et al.* (2005). Moreover, some European breeds have not proven any impacts of *MTNRIA* gene polymorphisms on their reproductive activities.

The difference might be well attributed to breed traits or body and environmental conditions. Thus, there is a need to expand the knowledge of the effects of *MTNRIA* gene polymorphism for the clarification of the mechanisms regulating the different reproductive responses of the different sheep breeds (Mura *et al.* 2014).

Association between *MTNRIA* gene polymorphism and birth and body weights

There are restricted opportunities for a selection within the breeds due to the fact that less heritability (nearly 0.05-0.15) occurs for most reproductive traits compared to many other traits.

Nonetheless, to optimize reproductive potentials, a within-breed selection must be done after crossing the divergent breeds to the genetic potentials rapidly reset for those traits, through which basic changes in litter size or seasonal breeding patterns are best achieved.

Another opportunity can be gained by quickly adjusting genetic potentials via various mutations that affect the ovulation rate and litter size of sheep though a careful breeding management is required, particularly for production traits (Notter, 2012).

Our results revealed that *MnlI* marker site in Shal × Romanov breeds had a significant effect ($P>0.05$) on a 3-month body weight (BW3) as ewes with MM genotypes had significantly higher BW3 than mm individuals. However, no significant differences were observed between MM and mm marker sites for 6-month birth and body weights (BW6) (Table 2). The same results were observed for Shal breed as MM and Mm genotypes were detected for BW, BW3, and BW6 traits (Table 3).

Table 2 Production performance (Mean±SEM) of Shal × Romanov breed

Traits	P-value	Genotype	
		MM	mm
BW	0.185 ^{ns}	4.38±0.13	4.75±0.23
BW3	0.034*	20.09±0.47	17.88±0.97
BW6	0.197 ^{ns}	28.06±0.73	26.02±1.45

BW: birth weight; BW3 and 6: body weight at 3 and 6 months of age.

* ($P<0.05$).

SEM: standard error of the means.

NS: non significant.

Table 3 Production performance (Mean±SEM) of Shal breed

Traits	P-value	Genotype	
		MM	Mm
BW	0.106 ^{ns}	4.02±0.25	3.51±0.11
BW3	0.013*	25.3±0.77	22.77±0.35
BW6	0.552 ^{ns}	37.3±1.18	36.61±0.40

BW: birth weight; BW3 and 6: body weight at 3 and 6 months of age.

* (P<0.05).

SEM: standard error of the means.

NS: non significant.

Although in our study, BW3 was affected by *MnlI* genotypes in both Shal and Shal × Romanov breeds, the production traits of Shal and Shal × Romanov breeds were not affected by *MnlI* genotypes based on BW and BW6 traits. Also, they were not influenced by mm or Mm genotypes in the current studies. In their study on the seasonal reproduction of Zandi breed, Hatami *et al.* (2014) reported that *MTNR1A/MnlI* marker site had a significant effect on BW1 as the ewes with Mm genotypes were found to have higher BW1 compared to MM individuals. No significant association was observed between *MTNR1A/MnlI* marker site and the other study traits, including BW, BW3, and BW6.

CONCLUSION

In the current research, the 2 genotypes of MM and Mm were detected in Shal population involved in seasonal reproduction. However, MM and mm genotypes were detected in Shal with Romanov crossbreeding. Thus, mm genotype observed as a measurement for litter size in sheep breeds successfully introduced into the crossbreeds. Therefore, based on BW and BW6 traits, the production traits of Shal and Shal × Romanov breeds were not affected by *MnlI* genotypes. Nevertheless, further studies are recommended to reveal the relationship between *MTNR1A* gene polymorphism at *MnlI* site and reproductive performance of Shal × Romanov ewes.

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