IJAS Iranian Journal of Applied Animal Science

Association between *MTNR1A* and *CYP19* Genes Polymorphisms and Economic Traits in Kurdi Sheep

Research Article

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Received on: 5 Aug 2016
Revised on: 4 Nov 2016
Accepted on: 15 Nov 2016
Online Published on: Mar 2017

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Online version is available on: www.ijas.ir

ABSTRACT

The ovine melatonin receptor 1A (*MTNR1A*) and aromatase (*CYP19*) genes were structurally characterized and the association between their variants and reproductive and growth traits was studied in Kurdi sheep at Kurdi sheep breeding station located in Shirvan, Iran. The genomic DNA was extracted by guanidine thiocyanate-silica gel method. Polymerase chain reaction was carried out to amplify 824 bp fragment of exon 2 of *MTNR1A* and 140 bp fragment of the exon 3 of the ovine *CYP19* genes. The PCR products were digested with restriction endonucleases RsaI for *MTNR1A* and *BstMBI* for *CYP19* genes and checked by polyacrylamide gel electrophoresis for the presence of restriction sites. Two alleles were found for all the loci investigated, which were named as A and B for *CYP19*, and R and r for *MTRN1A*. Allelic frequencies for *MTRN1A* were 0.49 and 0.51 for R and r alleles, while in the case of *CYP19* gene, frequencies were 0.475 and 0.525for A and B alleles, respectively. Association analysis did not show any significant relations between *MTNR1A* gene polymorphisms and litter size (LS), age at first lambing (AFL) and lambing interval (LI). Moreover, *CYP19* gene polymorphism did not affect birth weight (BW), weaning weight (WW), 6, 9 and 12 months (YW) body weights, age at first lambing (AFL) and lambing interval (LI).

KEY WORDS *CYP19* gene, growth traits, *MTNR1A* gene, polymorphism, reproductive traits.

INTRODUCTION

Aromatase is a cytochrome P450 enzyme complex that is encoded by the *CYP19* gene and catalyzes a critical reaction for estrogen biosynthesis involving the formation of aromatic C18 estrogens from C19 androgens. The cytochrome P450 aromatase (P450aro, CYP19) is a microsomal member of the cytochrome P450 superfamily (Nelson *et al.* 1993). The aromatase cytochrome P450 is necessary for the biosynthesis of estrogens in several tissues, most importantly ovaries, adipose tissue and brain. Estrogens play fundamental roles including endocrine, paracrine and autocrine activities involved in there gulation of male and female reproduction also in metabolic processes like fat deposition and growth (Heine *et al.* 2000; Jones *et al.* 2000; Simpson *et al.* 2000). The *CYP19* gene has been mapped to bands q24-q31 of chromosome 7 in sheep (Payen *et al.* 1995; Goldammer *et al.* 1999). In codon 69 which is located in exon 3, a silent C/T transition in several animals was found (Vanselow *et al.* 1999). Lôbo *et al.* (2009) have reported that in Brazilian sheep breeds, this polymorphism makes the differences in performance traits including litter weight, lambing interval, lambing age, reproductive and maternal ability. The melatonin pineal hormone (N-acetyl-5-

methoxytryptamine) occurs only during the hours of darkness which regulates circadian rhythms and reproduction changes in mammals with seasonally reproductive function (Reppert *et al*. 1994). The MLT (melatonin) can also be produced by extra-pineal sites like the retina, the gastrointestinal tract and the innate immune system (Jaworek *et al*. 2005). In mammals, two specific receptors sub types i.e. MT1 and MT2, encoded by the *MTNR1A* and *MTNR1B* genes, respectively. The MT1 and MT2 receptors are involved in the melatonin secretion, of which, only the melatonin receptor subtype 1A (*MNTR1A*) gene is considered to be a candidate gene and seems to play a key role in the control of photoperiod-induced seasonality mediated by the circadian concentrations of melatonin (Dubocovich *et al*. 1988; Weaver *et al*. 1996). The *MTNR1A* gene has been mapped to ovine chromosome 26, consists of two exons divided by a large intron (Reppert *et al*. 1994; Messer *et al*. 1997). Exon II of the gene encoding the MT1 receptor in sheep has two restriction fragment length polymorphism (RFLP) sites, one for *MnlI* and the second for *RsaI* enzyme (Messer *et al*. 1997). In sheep, the MT1 receptor encoded by exon 2 of the *MTNR1A* gene and this exon has two restriction fragment length polymorphism (RFLP) sites, one for *MnlI* and the second for *RsaI* enzyme (Messer *et al*. 1997). The structure and polymorphism of exon 2 of the *MTNR1A* gene using the *RsaI* restriction enzyme has been evaluated in several sheep breeds (Chu *et al*. 2003; Notter *et al*. 2003; Mateescu *et al*. 2009; Hristova *et al*. 2012; Martínez-Royo *et al*. 2009; Moradi *et al*. 2014). Melatonin acts as a natural inhibitor of the aromatase activity and expression by regulating the gene expression of specific aromatase promoter regions (Martınez-Campa *et al*. 2012). The geographic origins of the animals and photoperiod, with the intermediary activity of melatonin are important factors regarding the sheep reproductive activity through effecting on the aromatase activity (Mora *et al*. 2014). The objectives of the present study were first to detect the PCR-RFLP polymorphism of *MTNR1A* and *CYP19* genes and secondly to investigate the associations between *MTNR1A* and *CYP19* genes and growth and reproductive traits in Kurdi sheep.

MATERIALS AND METHODS

Genotyping

In this study, venous jugular blood samples (5 mL per ewe) were collected from 120 pure bred Kurdi ewes from Kurdi sheep breeding station located in Shirvan, Iran and transferred into vacutainer tubes containing 0.5 molar ethylene diamine tetracetic acid (EDTA) as anticoagulant and frozen at -20 ˚C. Genomic DNA was extracted from whole bloodusing a commercial kit (Diatom DNA Prep100, ISO Gene, Moscow) following the manufacturer's protocol.

The quantity and quality of the isolated DNA were determined using spectrophotometry and agarose gel electrophoresis. Polymerase chain reactions (PCR) were carried-out using Personal Cycler™ thermocycler (Biometra, Germany) and PCR Master Kit (Cinnaclon Inc., Iran). Master Mix consisted of 0.04 U/µL of TaqDNA polymerase, 10X PCR buffer, 3 m*M* $MgCl_2$ and 0.04 m*M* dNTPs (each). Each reaction mixture consisted of12.5 µL of the master mix, 1 µL of the DNA solution (50 to 100 ng/µL), 1 µL of each primer (5 pmol/µL) and some deionized water making up a final volume of 25 µL.

Amplification of a 140 bp fragment of the exon 3 of the ovine *CYP19* gene was carried out using primers (synthesized by CinnaGen, Iran) described by Vanselow *et al*. (1999), in agreement with the sequence deposited in GenBank (AJ012153):

CYP19-F (5'-CCA GCT ACT TTC TGG GAA TT-3')
CYP19-R (5'-AAT AAG GGT TTC CTC TCC ACA-3')

The amplification program consisted of an initial denaturation at 94 ˚C for 5 min followed by 35 cycles of denaturation at 94 ˚C for 30 sec, annealing at 55 ˚C for 30 sec, extension at 72 ˚C for 30 sec and a final extension at 72 ˚C for 5 min. For amplifying an 824 bp fragment of the main part of the exon 2 of the ovine *MTNR1A* gene with specific primers (synthesized by CinnaGen, Iran) as described by Messer *et al*. (1997), in agreement with the sequence deposited in GenBank (U14109):

MTNR1A-F(5'-TGTGTTTGTGGTGAGCCTGG-3')
MTNR1A-R(5'-ATGGAGAGGGTTTGCGTTTA-3')

The amplification reaction was carried-out under the following conditions: an initial denaturation step at 94 ˚C for 5 min followed by 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 58.5 ˚C for 1 min and extension at 72 ˚C for 2 min and a final extension of 72 ˚C for 5 min. Then, products of amplification were analyzed by 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized under ultraviolet light. A 10 µL of PCR products were incubated for 14h at 37 ˚C with 1 µL (10 units) of *BstMBI* and *RsaI* enzymes for Cyp19 and *MTNR1A* genes, respectively. The digestion products were also electrophoresed on 8% acrylamide gel and visualized in parallel with a 50 bp DNA marker.

Statistical analysis

Determination of genotypic and allelic frequencies and Hardy-Weinberg (H-W) equilibrium test were carried out using Pop-Gene software (V 1.31) (Yeh *et al*. 1997).

In order to test the association of different conformational patterns with the studied traits, statistical analysis was performed using general linear model (GLM) procedure of the SAS program and least squares means of the banding patterns were compared using the Tukey-Kramer test at 5 percent probability level (SAS, 2000).

Studied traits were growth and reproductive traits including birth weight (BW), weaning weight (WW), 6, 9 and 12 (YW) month weights, age at first lambing (AFL) and lambing interval (LI).

The Following models were used for growth and reproductive traits, respectively:

$$y_{ijklm} = \mu + G_i + A_j + B_k + T_l + e_{ijklm}$$
$$y_{ijklmn} = \mu + G_i + YC_j + MC_k + A_l + YB_m + e_{ijklmn}$$

Where:

y_{ijklm} and y_{ijklmn}: growth and reproductive traits, respectively.

μ: overall mean.

G_i: fixed effect of the i^{th} banding patterns (i=1,2,3).

A_j: fixed effect of the j^{th} dam age (j=1,...,8).

B_k: fixed effect of the K^{th} year.

T_l: fixed effect of the l^{th} birth type.

YC_j: fixed effect of the j^{th} lambing year.

MC_k: effect of the k^{th} lambing season.

YB_m: effect of m^{th} birth year.

e_{ijklm} and e_{ijklmn}: random residual errors.

RESULTS AND DISCUSSION

A 140 bp fragment of the ovine cyp19 gene from exon 3 was amplified successfully. *BstMBI* restriction enzyme was used to digest the PCR products. The PCR digestion products of 120 samples showed only two genotypes: AB and BB. AB genotype exhibited 140, 82 and 58 bpfragments and BB genotype had only one fragment, 140 bp (Figure 1) which was in agreement with Vanselow *et al.* (1999) and Lôbo *et al.* (2009) reports. The frequencies of individual alleles and genotypes in the present study are shown in Table 1.

The frequency of B (0.525) was higher than allele A (0.475) and frequency of AB (0.95) was higher than BB genotype. No significant relation (P>0.05) was found between cyp19 conformational patterns and all the studied traits in the population (Table 2). In study of Vanselow *et al.* (1999) on five breed groups of European sheep, the allele frequencies were 0.74 for allele A and 0.26 for allele B in Hungarian Merino sheep (n=38), in Awassi, Tsigaja, Brith Milk sheep (0.6 for allele A and 0.4 for allele B; n=5) and for Lacaune breed, a frequency of 1.0 for allele A and zero for allele B (n=5).

In another study on several breed groups, a greater frequency of allele B was observed in the Brazilian Somali (1, n=13), Poll Dorset (0.61, n=9) Santa Inês (0.6, n=71) and 1/2 Dorper (0.8, n=18).

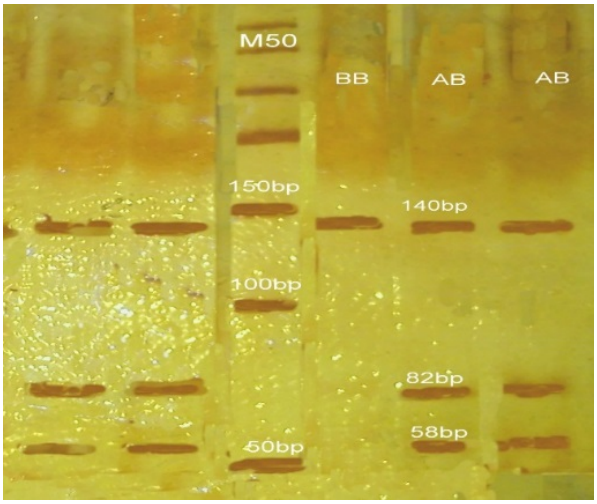

Figure 1 Analysis of RFLP polymorphism of aromatase gene (*Cyp19*) in Kurdi sheep. Non-digested PCR products are 140 bp in size (allele B). In the case of allele A, there were two fragments of 82 bp and 58 bp, respectively
PM: 50 bp molecular weight ladder
AB and BB: deduced genotypes

Table 1 Observed alleles and genotypic frequencies for *CYP19* gene in Kurdi sheep

Allele frequencies		Genotype frequencies		
A	B	AA	AB	BB
0.475	0.525	0	0.95	0.05

In the studied population, AA genotype was not observed and results indicated a relation between the genotypes and some growth and reproductive traits, so that, lower age at first lambing in all 1/2 Dorper BB and lower lambing interval in Santa Inês BB ewes and higher litter weight at weaning for AB ewes (in same genetic groups) were observed (Lôbo *et al.* 2009). Mora *et al.* (2014), investigated C242T polymorphism at the Cyp19 gene in four breed groups composite of Texel, Dorper, White Dorper and Santa Inês and three distinct genotypes: AA, AB and BB were observed. In their study, the Texel sheep group with European origin had highest frequencies for allele A but highest frequencies of allele B was observed in White Dorper sheep originated from tropical countries. Results suggested a relation between the higher frequency of alleles A and B with the ancestral geographic origin of the sheep. In agreement with the results of the present investigation, allele B frequency in Brazilian Somali, Poll Dorset, Santa Inês and 1/2 Dorper sheep (Lôbo *et al.* 2009) and Dorper , White Dorper and Santa Inês (Mora *et al.* 2014) was higher than allele A.

Table 2 Least square means of studied traits for the *CYP19* gene

Trait	Genotypes		F-statistic	P-value
	AB	BB		
BW (kg)	4.49±0.07	4.86±0.42	1.06	0.35
WW (kg)	26.78±0.36	25.08±1.93	0.98	0.37
6MW (kg)	29.59±0.41	28.60±2.35	0.10	0.90
9MW (kg)	41.73±8.12	43.43±49.91	0.05	0.95
YW (kg)	41.41±0.53	43.25±3.05	0.49	0.61
AFL (d)	676.82±10.58	662.36±30.14	0.07	0.93
LI (d)	353.94±3.92	357.54±11.08	0.35	0.70

BW: birth weight; WW: weaning weight; 6MW: 6-month weight; 9MW: 9-month weight; YW: yearling weight; AFL: age at first lambing and LI: lambing interval.

But in Texel sheep (Mora *et al*. 2014) and Hungarian Merino, Awassi, Tsigaja, Brith Milk and Lacaune sheep (Vanselow *et al*. 1999) higher frequency of allele A was found. Also, there were no animals with AA genotype in our study, probably due to the low frequency of allele A which was in agreement with the results obtained by Vanselow *et al*. (1999) and Lôbo *et al*. (2009), but disagree with the results found by Mora *et al*. (2014). Different genotypes for the cype19 gene among sheep produced a differences in some reproductive and growth traits (first lambing and lambing interval, weight at birth and at weaning, and daily weight gain) (Lôbo *et al*. 2009).

In the present study, no significant association was found between genotypes and the studied traits in Kurdi sheep. Apart from above, this locus did not show Hardy-Weinberg equilibrium. This approves that factors leading to disequilibrium, especially selection, may influence the genetic structure of the population. Exon 2 of *MTNR1A* gene with 824 bp length was amplified. *RsaI* restriction enzyme recognizes and cuts the PCR products. For *RsaI*, four cleavage sites (53 bp, 267 bp, 23 bp, 411 bp and 70 bp) within the amplification fragment was found but only one fragment was polymorphic (Chu *et al*. 2003).

This site was at 604 positionin the reference sequence (Reppert *et al*. 1994). Digestion of 120 samples with *RsaI* revealed three genotypes i.e. RR (411 bp/267 bp), Rr (411/290 bp/267 bp) and rr (411 bp/290 bp) in Kurdi sheep (Figure 2).

These results were consistent with those of Notter *et al*. (2003), Chu *et al*. (2006) and Martínez-Royo *et al*. (2009), while the rr genotype was not found in local Karnobatska breed (Hristova *et al*. 2012). Furthermore, for Chios, White Karaman and Awassi breeds, only one genotype (rr) was detected and no polymorphism at the *RsaI* cleavage sites was founding three sheep breeds (Şeker *et al*. 2011). Frequencies of individual alleles and genotypes in the present study are shown in Table 3. In the present study, frequencies of RR, Rr and rr genotypes were 0.275, 0.5 and 0.275, respectively which were similar to those recorded in German Mutton Merino ewes (0.24 RR, 0.48 Rr and 0.28 rr) by Chu *et al*. (2006).

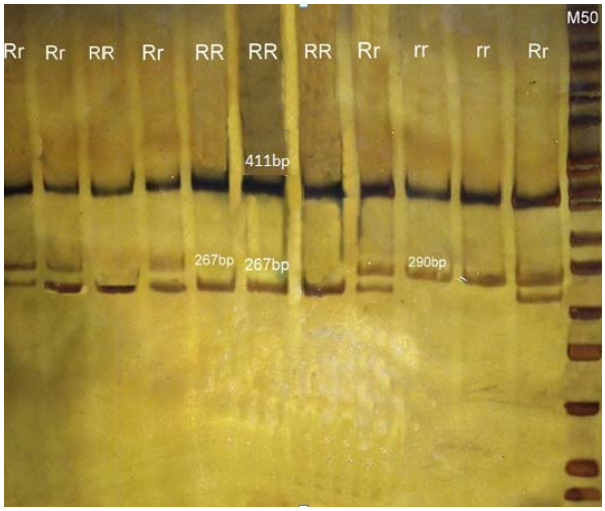

Figure 2 Analysis of RFLP polymorphism exon 2 of the *MTNR1A* gene in Kurdi sheep. Three genotypes: RR (411 bp/267 bp), Rr (410/290 bp/267 bp) and rr (411 bp/290 bp) were detected
PM: 50 bp molecular weight ladder

Table 3 Observed alleles and genotypic frequencies for *MTNR1A* gene in Kurdi sheep

Allele frequencies		Genotype frequencies		
R	r	RR	Rr	rr
0.49	0.51	0.275	0.45	0.275

Chu *et al*. (2006), determined allele and genotype frequencies of *MTNR1A* gene in non-seasonal estrous breeds (Small Tail Han, Hu ewes) and in seasonal estrous breeds (Suffolk, Dorset and German Mutton Merino ewes). A frequency of RR genotypes was higher, and frequency of rr genotype was lower in non-seasonal estrous sheep breeds than in seasonal estrous sheep breeds. Moreover, they detected a relation between rr genotype and seasonal estrus in ewes and association between RR genotypes and non-seasonal estrus in ewes, while in the Rasa Aragonesa breed rallele of SNP606/*RsaI* of *MNTR1A* gene was associated with a higher percentage of oestrous cyclic ewes. These findings, indicated that other genes closely linked or regulatory sequences of the *MNTR1A* gene could be influencing the ability to breed out of season (Martínez-Royo *et al*. 2009).

Table 4 Least square means of studied traits for the *MTNR1A* gene

Trait	Genotypes			F-statistic	P-value
	RR	Rr	rr		
AFL (d)	678.5±15.43	675.96±16.33	678.58±16.18	1	0.39
LI (d)	351.94.5±5.94	352.74.5±5.80	355.50±6.06	0.55	0.65
LS (d)	1.13±0.14	1.16±0.14	1.07±0.14	1.08	0.35

AFL: age at first lambing; LI: lambing interval and LS: litter size.

In the present study, no significant relation (P>0.05) was found between *MTNR1A* conformational patterns and the studied traits in Kordi sheep (Table 4). Similar to these findings, in the study of Notter *et al*. (2003), genotypic effects on litter size were small and not significant, while Chu *et al*. (2003) identified a relation between the *MTNR1A* gene and litter size of ewes at second lambing seasonal and highly prolific Han sheep. The local populations of Bulgarian sheep breeds, Starozagorska, Karnobatska, Breznishka and Sofiiska (Elin-Pelinska) were characterized by frequency of the R allele: 0.302, 0.729, 0.520, 0.526 and r allele: 0.698, 0.271, 0.480, 0.474 and respectively. These findings confirmed the importance of MTNR1A gene as a potential DNA marker in marker – assisted selection (Hristova *et al*. 2012).

The present study should be considered as preliminary investigation and further research is needed to provide better distinguishing function of *MTRN1A* and *CYP19* genes and determination of their effects on economic traits of Kurdi sheep.

CONCLUSION

Genetic polymorphism was approved for *MTNR1A* and *CYP19* genes in Kurdi sheep. No significant association between the polymorphisms of these genes with reproductive and growth traits was found. Further researches with more number of observations are needed for more reliable association study.

ACKNOWLEDGEMENT

This work was supported by by Gorgan University of Agricultural Science and Natural Resources, (Golestan, I.R. Iran). The authors are also thankful to the staff of Hoseianabad sheep breeding station, Shirvan, Iran, for their great help to provide blood samples and data.

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