# Genetic polymorphism at *MTNR1A*, *CAST* and *CAPN* loci in Iranian Karakul sheep

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***Abstract***
*Genotypes for melatonin receptor type 1A (MTNR1A) and Calpastatin (CAST) were determined by enzymatic digestion of PCR products and Calpain(CAPN) genotype detected by PCR-SSCP method in Iranian Karakul sheep. Blood samples were collected from 100 purebred Karakul sheep. The extraction of genomic DNA was based on guanidinium thiocyanate-silica gel method. PCR amplicons were digested with restriction enzymes MnlI and MspI for MTNR1A and CAST genes, respectively. The MTNR1A locus had two alleles with frequencies of 0.79 for (+) and 0.21 for (-) alleles. Allelic frequencies for CAST locus were 0.85 for M and 0.15 for N. In addition, Calpain had two alleles A and B with respective frequencies of 0.79 and 0.21. The observed heterozygosity values for MTNR1A, Calpastatin and Calpain locus were 0.42, 0.29 and 0.35, respectively. The $X^2$ test confirmed the existence of Hardy-Weinberg equilibrium for the three loci in the population. The data showed a large variation in studied genes. The genetic polymorphism could be regarded as useful tool for selection programs based on marker- assisted selection between different genotypes of those loci.*
***Keywords****: MTNR1A; Calpastatin; Calpain; Polymorphism; PCR-RFLP; SSCP; Karakul Sheep.*

## INTRODUCTION

Several DNA polymorphisms have been considered as potential tools for selection programs in domestic animals. DNA-based molecular methods have made a potentially more efficient and flexible selection tool for genotyping of animals of any age and sex for milk, reproduction and meat genes. Selection efficiency, however, depends on allelic frequencies, the effect on these polymorphisms on dairy, reproduction and meat traits and their technological properties. The genetic polymorphisms of MTNR1A, Calpastatin and Calpain have been performed in sheep (Pelletier *et al*., 2000; Notter *et al*., 2003; Palmer *et al*., 1999a).

Seasonal changes in reproductive activities are common among various mammalian species of temperate latitudes (Ortavant *et al*., 1985). The seasonality of reproductive activity in sheep breeds in temperate latitude is controlled by photoperiod (Thiery *et al*., 2002). The photoperiodic information is conveyed through several neural relays from the retina to the pineal gland where the light signal is translated into daily cycle of melatonin secretion (Malpaux *et al*., 1999) and its level is high at night and low during day time (Ganguly *et al*., 2002).

The length of nocturnal secretion of melatonin reflects the duration of night and it regulates the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus. Changes in GnRH release induce corresponding changes in Luteinizing Hormone (LH) secretion which is responsible for the alternating presence or absence of ovulation in the female and varying sperm production in the male (Malpaux *et al*., 1999). Two polymorphic RFLP sites within the ovine melatonin receptor1a gene (*MTNR1A*) were observed by Messer *et al*. (1997). A particular allelic form of the $Mel_{1a}$ receptor genies cauterized by the absence of a *Mnl*I restriction site at position 605 of the coding sequence, leading to (-/-) homozygous genotypes when the corresponding mutation occurs on both chromosomes (Migaud *et al*., 2002).

The intensively investigated genes in farm animal are Calpastatin (*CAST*) and Calpain (*CAPN*). Calpastatin and Calpain deserve special attention because of their major role in meat production (Chung

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*et al*., 1999). The Calpain-Calpastatin system (CCS) comprises a family of calcium dependent neutral proteases. The Calpastatin is specific inhibitor of the Calpain which regulates their *in vivo* activity (Chung *et al*., 1999). Calpastatin is a multi headed inhibitor and capable of inhibiting more than one calpain molecule. Each inhibitory domain of calpastatin has three subdomains. A, B, and C; A binds to domain IV and C binds to domain VI of the calpains (Amanda *et al.,* 2006). The CCS is found in most animal tissues and influences many important processes including muscle development and degradation, meat tenderization postmortem and cataract formation (Chung *et al*., 1999). The Calpains have been shown to play the major role in post-mortem tenderization in beef, lamb, and pork by degrading specific muscle structural proteins. Using a molecular genetic approach to study meat quality in sheep, Palmer *et al*. (1999a) have chosen the ovine *CAST* as a candidate gene for meat quality. They have described a two-allele system of polymorphic variant (M and N) in a region of the ovine *CAST* (exon and intron regions from a portion of the first repetitive domain) by PCR-RFLP method. Since 1995, Palmer *et al*. (1999a) have carried out slaughter trials on small groups of Dorset Down Hogget's and Dorset Down × Coopworth lambs to ascertain any association between meat quality traits and molecular markers in *CAST*. Sheep with the genotype ac for *CAST* locus (in PCR-SSCP method) were compared for traits indicated an association with increased live weight gain (12-17%, $P<0.05$), increased age-corrected carcass weight (15-18%, $P<0.05$), but increased shear force (4-12%, no significant) compared to sheep with the *CAST* genotype aa (Palmer *et al*., 1999b). A number of studies have shown that the Calpain system is also important in normal skeletal muscle growth (Chung *et al*., 2002). Increased rate of skeletal muscle growth can result from a decreased rate of muscle protein degradation and this is associated with a decrease in activity of the Calpain system, due principally to a large increase in Calpastatin activity. It is now accepted that Calpain-mediated degradation of myofibrillar proteins is responsible for the post-mortem meat tenderization, which occurs during storage at refrigeration temperatures (Chung *et al*., 2002). Calpastatin is the endogenous and specific inhibitor of Calpains, which regulates the rate and extent of post-mortem tenderization (Kocwin *et al*., 2003).

The aim of this study was to investigate the polymorphism at *MTNR1A*, Calpastatin and Calpain genes in Karakul sheep as the fur-type native breed in North-East of Iran using PCR-RFLP and PCR-SSCP methods.

## MATERIALS AND METHODS

**Animals and DNA extraction:** Blood samples were randomly collected from 100 purebred Karakul sheep from Karakul Breeding Station located at Sarakhs city in North East of Khorasan province in Iran. Three ml of blood was collected by EDTA-anticoagulated vacutainer tube via venipuncture from jugular vein. DNA was extracted from 100 µl of blood as described by Boom *et al*. (1989). Quality and quantity of DNA was assessed by taking the optical density at wavelength of 260 and 280 nm, respectively.

**PCR:** The PCR was run in a 25 µl reaction using the Biometra T-Personal Ver: 1.11 thermocycler. The PCR mixture contained: 50-100 ng of extracted DNA, 2.5 µl of 10X PCR buffer (200 mM $(NH_4)_2SO_4$, 0.1 mM Tween 20%, 750 mM Tris-HCl pH 8.8), 2.5 mM $MgCl_2$, 200 µM dNTPs, and 3 µl mix of oligonucleotids (10 pmol from each primer), 1U *Taq* DNA polymerase and 11 µl $ddH_2O$. the amplification conditions for each locus was follow:

*MTNR1A*: Amplification of *MTNR1A* was done by the primers of Messer *et al*. (1997). The amplification was done for 35 cycles at the following program: denaturation at 94ºC for 5 min, annealing at 59ºC for 1 min and extension at 72ºC for 2 min and ended in the final extension time for 10 min.

*CAST*: The Amplification was done for thirty-five cycles of 95ºC (1 min), 62ºC (1 min), and 72ºC (2 min) followed by final extension 72ºC (8 min). The Exon (1C, 1D) of the ovine Calpastatin gene (*CAST* 1) was amplified to a product of 622 bp fragment using primers based on the sequences bovine Calpastatin gene. Primer sequences were ovine 1C: 5´-TGG GGC CCA ATG ACG CCA TCG ATG-3´ and ovine 1D: 5´-GGT GGA GCA GCA CTT CTG ATC ACC-3´ (Gene Bank accession No. AY834765).

*CAPN*: PCR program included a preliminary denaturizing at 95ºC, followed by 35 cycles, denaturing at 94ºC for 45 sec annealing at 59ºC for 1 min, extension at 72ºC for 1.5 min and 10 min at 72ºC as final extension. Amplification of Calpain gene was done with primers (CAPN456F: 5´-AAC ATT CTC AAC AAA GTG GTG-3´, CAPN456R: 5´-ACA TCC AT ACA GCC ACC AT-3´) which designed according to the published bovine nucleotide cDNA sequence (Gene Bank accession No. J05065). Products of amplification were recognized by electrophoresis on 1.5% agarose gel which was stained by etidium bromide.

**RFLP analysis:** 5 µl of each PCR products were incubated separately for 5 h at 37ºC with 5 U of *Mnl*I and *Msp*I enzymes for *MTNR1A* and Calpastatin genes, respectively. Digestion products of *MTNR1A* were separated by electrophoresis on 8% non-denaturant polyacrylamid gel and visualized after silver staining. Digestion products of Calpastatin were separated by electrophoresis on 2% agarose gel which was stained by etidium bromide.

**SSCP analysis:** For the genotyping of Calpain locus, PCR products were diluted with 12 µl of running buffer, containing 800 µl formamid, 100 µl bromophenol blue 1%, 100 µl xylenecyanol 1%, 2 µl 0.5 M EDTA and 1 µl 10 M NaOH. After heating at 95ºC for five minutes, the samples were immediately placed on ice and polymorphisms were detected using 8% non-denaturing polyacrylamid gel with 10 % glycerol. The mixture was electrophoresed for 3-4 h at 250 V and 10°C and DNA fragments were visualized using silver staining method.

**Statistical analysis:** The observed and expected heterozygosities of genotypic and allelic frequencies, and test for Hardy-Weinberg equilibrium (HWE) were calculated using PopGene32 (Ver. 1.31) program (Yeh *et al.,* 1999).

## RESULTS

The three loci were polymorphic in Karakul sheep. The genotypes (+/+) and (+/-) for *MTNR1A*, MM, MN and NN for Calpastatin and AA and AB for Calpain locus were observed. Table1 shows the allelic frequencies for three loci in the Iranian Karakul sheep.

**Analysis of *MTNR1A* locus:** An 824 bp fragment of the ovine *MTNR1A* gene from exon II was amplified successfully. Digestion with restriction endonuclease *Mnl*I differentiate (+) and (-) alleles. The (+/+) genotype exhibited both fragments of 236 and 67bp. The (+/-) genotype exhibited 303, 236, and 67-bp. The (-/-) genotype was not seen in this study (Fig. 1). The *MTNR1A* locus had two alleles with frequencies of 0.79 and 0.21 for (+) and (-) alleles, respectively. The frequency of genotype (+/+) was 0.70 and genotype (+/-) 0.30 (Fig. 4).

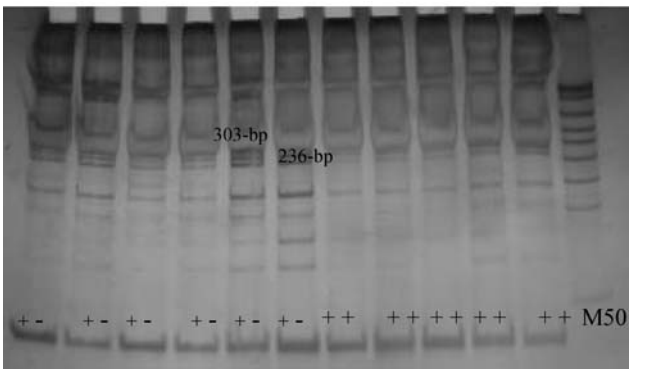


**Figure 1.** Digestion of PCR products with *Mnl*I. (Molecular marker was M50 (500, 450, 400, 350, 300, 250, 200, 150, 100, and 50 bp).

**Analysis of Calpastatin locus:** A 622 bp fragment was amplified from *CAST*. Digestion with restriction endonuclease *Msp*I differentiated two M and N alleles. The MM genotype exhibited 2 fragments of 336 and 286 bp, MN genotype exhibited 622, 336 and 286 bp fragments and NN genotypes exhibited a 622 bp fragment (Fig. 2). The allelic frequencies were 0.79 and 0.21 for M and N, respectively. The genotypic frequencies for MM, MN and NN in Karakul sheep were 0.61, 0.36 and 0.03, respectively (Fig. 4).

**Table 1.** Allelic frequencies, observed heterozygosity (Obs-Het) and expected heterozygosity (Exp-Het), average heterozygosity and Nei values for MTNR1A, CAPN and CAST loci.

| Locus | Alleles | | Obs_Het | Exp_Het[1] | Nei[2] | Ave_Het |
|---|---|---|---|---|---|---|
| | 1* | 2** | | | | |
| *MTNR1A* | 0.79 | 0.21 | 0.42 | 0.33 | 0.50 | 0.25 |
| *CAPN* | 0.85 | 0.15 | 0.29 | 0.25 | 0.25 | 0.25 |
| *CAST* | 0.79 | 0.21 | 0.35 | 0.67 | 0.33 | 0.33 |

[1]Expected heterozygosity computed using Levene method (1949) [2]Nei's (1973) Expected heterozygosity *The abbreviations of (+) for *MTNR1A* allele, (A ) for *CAPN* allele and ( M ) for *CAST* allele **The abbreviations of (-) for *MTNR1A* allele, (B ) for *CAPN* allele and (N ) for *CAST* allele.

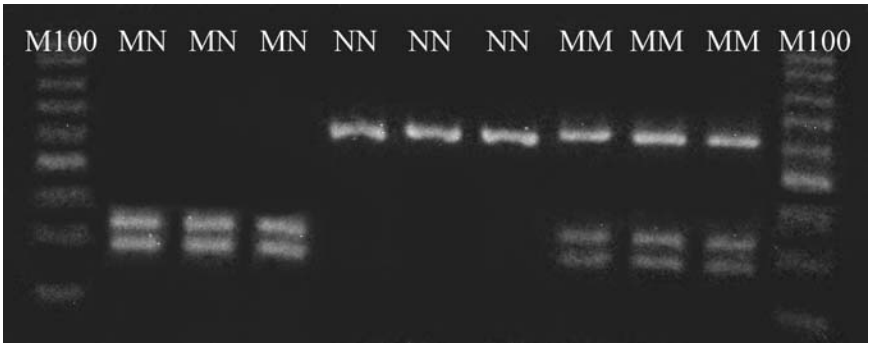


**Figure 2.** *Msp*I Digestion of PCR products. (Molecular marker is M100 (1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100bp).

Average heterozygisity for this locus was low. Observed heterozygosity, expected heterozygosity, Nei's expected heterozygosity and average heterozygosity of Calpastatin locus and Calpain were low.and medium, respectively.

**Analysis of Calpain locus:** The ovine Calpain II regulatory gene, exon 5 and 6 including intron (CAPN456), was amplified and produce a 190 bp fragment. Under the SSCP analysis two genotypes of AA and AB with frequencies of 0.79 and 0.21 were observed, respectively (Fig. 3). In addition, Calpain had two alleles with frequencies of 0.79 for A and 0.21 for B (Table 1).

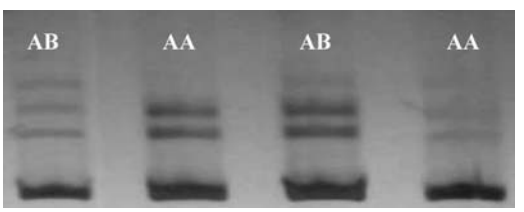


**Figure 3.** SSCP Patterns for Calpain gene in Karakul sheep breed.

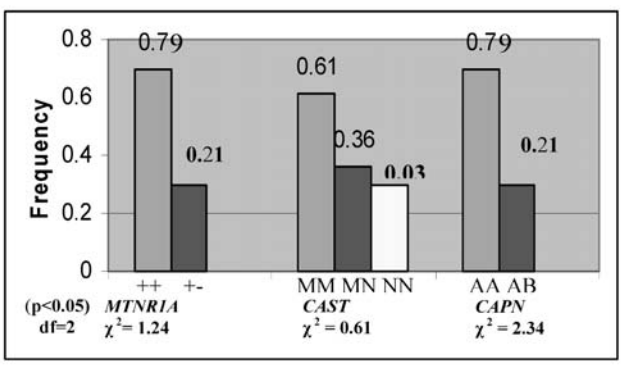


**Figure 4.** Genotypic frequencies of *MTNR*, *CAPN* and *CAST* loci.

## DISCUSSION

The observed frequencies for (+) and (-) variants of MTNR1A gene in this study were similar to the reported values by Pelletier *et al*. (2000) in Merino d›Arles ewes. The reported frequencies for (+) and (-) alleles by Ghiasi (2005) in Iranian Shall sheep breed were 0.79 and 0.21, respectively, which were similar to obtained data in this investigation. He Also found a genotypic frequency of 0.55 for (+/ +) genotype. Same as Ghiasi report (2005), (-/-) genotype was not detected in this study. In a ewes population (Three way crosses of 50% Dorset, 25% Rambouillet, and 25% Finnish Landrace) and selection program for improving reproduction efficiency and autumn lambing, Notter *et al.* (2005) measured this frequencies 0.42 for M (+) and 0.58 m (-) alleles. Average heterozygisity for this locus was as low as 0.25. Ghiasi (2005) also found a heterozygosity of 33% in Shall sheep. In accordance with the results of this experiment a high degree of Calpastatin locus polymorphism was reported in Dorset Down, Hoggets, Dorset Down × Coopworth sheep, Corriedale rams, Angus bulls, crossbred steer and pigs (Chung *et al*., 1999; Kocwin *et al.,* 2003; Palmer *et al.,* 1999a). According to Palmer *et al*. (1999b), the Calpastatin genotypes of MM, MN and NN were detected in Corridale rams and allelic frequencies were 77% for M and 12% for N alleles, which was in agreement with our results. In contrast, Elyasi *et al*. (2005) reported that the frequency of M allele was 0.50 in Ghezel × Arkharomerino sheep. They observed a high percentage of MN genotype in Arkharmerino (47.62%) and Gheze × Arkharomerino (46.67%). Nassiry (2005) reported a frequency of 0.88 for M and 0.12 for N alleles in Iranian Kurdi sheep. They found MM genotype with high frequency (0.76). The Nei's expected heterozygosity and average heterozygosity of Calpastatin locus for Karakul sheep were 0.33 and 0.33 respectively.

Palmer *et al*. (1999b) described a three alleles system of polymorphic variants (a, b and c) by PCR-SSCP in a region of the ovine *CAST*. However in an experiment with Angus bulls Palmer *et al*. (1999a) observed AA, AB and BB genotypes for *CASTI* and *CAST5* loci and AA, BB, CC, AB, AC and BC for *CAST10* locus. Single nucleotide polymorphisms (SNPs) in the calpastatin (CAST) gene was studied in *Bos taurus* (Jersey × Limousin, Angusand Hereford-cross cattle) cattle by Morris *et al.* (2006). Schenkel *et al.* (2006) was identified a SNP in the CAST gene (a G to C substitution) and genotyped on crossbred commercially fed heifers , steers, and bulls from beef feedlots, and steers from a University of Guelph feeding trial. They identified three genotypes (CC, CG and GG) for this SNP and reported that the *CAST's* SNP allele C was more frequent (63%) than G allele. Kurly *et al.* (2002) identified the polymorphism of Calpastatin gene with three restriction enzymes (*Hinf* I*, Msp*I*, Rsa*I) in Stambeek (Dutch large whith × Dutch Landrrace) pig breed. Palmer *et al.* (1999b) described a three alleles system of polymorphic variants (a, b and c) by PCR-SSCP in a region of the ovine *CAST*. Tahmorespour (2005) reported a gene frequency of 0.22 for c allele of *CAST* in Baluchi sheep by PCR-SSCP method, which was in agreement with the growth of purebred Dorset Down Lambs and Dorset Down × Coopworth Lambs (Palmer, 1999a).

The Calpain gene was investigated as a potential candidate gene for quantitative trait locus (QTL) affecting meat tenderness (Koohmaraie *et al*., 1992). Under a SSCP analysis and in a gel electrophoresis on non-denaturing condition, allelic frequencies of A and B allels were 0.85 and 0.15, respectively. Opposite results were reported by Tahmoorespour (2005), who found A and B with respect frequency value of 0.56 and 0.44 for *CAPN* in Baluchi sheep. Chung *et al*. (2000) analyzed the same exon with the same methodology and primers and found frequencies of 0.69 for A and 0.31 for B alleles. The genotypic frequencies of AA, AB and BB of *CAPN* genotypes in Karakul sheep were 0.70, 0.30 and 0.00 respectively. The observed heterozygosity (29.63%), expected heterozygosity (25.4%), Nei's heterozygosity (25.24 %) of *CAPN* locus for Karakul sheep were medium.

According to available data, the Karakul sheep is excellent source of biological information for studies on genetic characterization in Iran. This breed is kept in a closed station with no selection for these loci; therefore funding Hardy-Weinberg equilibrium for three loci. Karakul sheep showed a low degree of genetic variability for the *MTNR1A*, Calpastatin and Calpain loci. This can be attributed to the specific application of breeding methods, in this scientific center. In this station, a few male lambs are selected for breeding programs every year. With respect to the limited number of rams in breeding programs, lower value of heterozygosity and genetic variability is logically expected. Ram changing between different flocks can be an applicable method for lowering this adverse effect. Although low variables were found for the mentioned loci, on the other hand, these data provide on evidences that Iranian's Karakul sheep is a good polymorphic source for *MTNR1A*, Calpastatin and Calpain loci, which opens interesting prospects for future selection programs, especially marker-assistant selection for gain and meat traits.

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