# Allelic Polymorphism of Makoei Sheep Calpastatin Gene Identified by Polymerase Chain Reaction and Single Strand Conformation Polymorphism

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### ABSTRACT

Calpastatin (*CAST*) is a specific inhibiter of calpains, playing a role in meat tenderization and myogenesis. In the present study, the polymorphism of the *CAST* gene of Makoei sheep was investigated by polymerase chain reaction and single strand conformation polymorphism technique (PCR–SSCP). Genomic DNA was extracted from whole blood samples collected from 100 sheep. A 622 bp *CAST* exon 1 segment was amplified by standard PCR, using the locus specific primers. PCR products were subjected to a non-denaturing gel electrophoresis. Four SSCP patterns, representing four different genotypes, were identified. The frequencies of the observed genotypes were 0.31, 0.04, 0.63 and 0.02 for *AA*, *BB AB* and *AC*, respectively. Allele frequencies were 0.6313, 0.3586 and 0.01 for *A*, *B* and *C*, respectively. The Observed heterozygosity ( $H_{obs}$ ) value for *CAST* gene was 0.4728. The chi-square test showed significant (P< 0.01) deviation from Hardy-Weinberg equilibrium for this locus in Makoei sheep population.

Keywords: CAST gene, Makoei sheep, PCR, SSCP.

#### **INTRODUCTION**

The improvement in meat quality is the main goal of livestock production. Meat tenderness is one of the most important factors for quality assessment of the meat. The calpain proteolytic system has been identified as a factor for postmortem meat tenderization process through the proteolysis of myofibrillar and associated proteins (Koohmaraie, 1992; Taylor *et al.*, 1995). Variation in meat tenderness is due to the genetic variation, biological and physiological differences during slaughter,

and chemical differences during postmortem aging (Koohmaraie 1996).

Calpastatin (CAST) gene located on the fifth chromosome of sheep encodes a specific inhibitor calpain that plays important roles in the formation of muscle, degradation, and meat tenderness after slaughter (Gabor et al., 2009; Palmer et al., 1999). Polymorphisms in the bovine (Casas et al., 2006; Schenkel et al., 2006) and pigs (Ciobanu et al., 2004) CAST gene have been associated with meat tenderness, making the CAST gene an excellent candidate for controlling meat traits in livestock.

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Genetic polymorphism identification of the CAST gene and its relation to the meat quality could be used as a tool to predict meat tenderness in animals allowing breeders to enhance the trait (Seiler, 1994). In addition, genotyping animals bv employing this molecular marker will help to classify carcasses based on eating quality before slaughter (Lonergan et al., 1995). It was demonstrated that the favorable effect of the variants of CAST gene on pig carcass quality traits depends on the cut. It was also reported that post-mortem changes in different periods depends on the CAST/RsaI genotype. It seems that the BB genotype is related to the rate of glycolysis immediately after slaughter while the AA genotype is related to the rate of glycogenolysis in the process of muscles conversion into meat (Krzecio et al., 2008).

Palmer et al. (1998) have described two allelic systems of polymorphic variants (M and N) in a region of the bovine CAST by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. Using a molecular genetics approach to study meat quality in sheep, Palmer et al. (1999) chose the ovine CAST gene as a candidate gene for meat quality. A three allelic system of polymorphic variants (a, b, and c) have also been described by PCR and single strand conformation polymorphism (PCR-SSCP) in a region of the ovine and cattle CAST (Chung et al., 1999; Palmer et al., 2000).

The present study aimed to evaluate the genotype and gene frequencies at the ovine *CAST* gene of "Makoei" sheep breed in west Azerbaijan Breeding Station, Iran.

#### MATERIALS AND METHODS

## Sheep Blood Sample Collection and Genomic DNA Extraction

Makoei sheep examined in this study were fat-tailed sheep with medium body size and white color with black spots on face and feet. They are raised in East and West

Azerbailan Provinces of Iran and their main products are meat and wool (Saadat-Noori and Siah-Mansoor, 1992). Blood samples (approximately 2-3 ml) were obtained from 100 unrelated Makoei sheep from different parts of West Azerbaijan province and stored in EDTA-coated tubes. Genomic DNA was extracted from 0.3 ml of blood using the genomic DNA purification kit (Fermentas, EU) according to manufacturer's instructions. Quality and quantity of extracted DNA was measured by agarose gel (0.8 %) electrophoresis.

## Amplification of the Exon 1 of CAST Gene

The DNA amplification of the CAST gene was achieved by PCR. Two primers exon 1C (5'-TGGGGCCCAATGACGCCATCGATG-37) (5) and 1D exon GGTGGAGCAGCACTTCTGATCACC-3) targeting a fragment of 622 bp was employed as described by Palmer et al. (1998). The PCRs were carried out in 50 µl using PCR mastermix volumes kit (Cinnagen, Iran) containing 2.5 units Taq DNA Polymerase in reaction buffer, 4 mM MgCl<sub>2</sub>, 50 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer and about 100 ng of extracted DNA as template. Amplification was performed in Mastercycler (Eppendorf, Germany) using 35 cycles of incubation at 95°C for 45 seconds, 62°C for 1 minUTE, and 72°C for 75 seconds, with a final extension at 72°C for 7 minutes.

## Single Strand Confirmation Polymorphism (SSCP)

PCR products were mixed with 8  $\mu$ l of denaturing loading dye [95% (w/v) deionized formamide, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue and 0.02 M EDTA] in a total volume of 15  $\mu$ l. The mixture was denatured at 95°C for 5 minutes and was snap chilled on ice (Pipalia

*et al.*, 2004). The total volume was applied in a 15% polyacrylamide gel, as described by Herring *et al.* (1982). The electrophoresis was performed in 0.5 X TBE buffer (Tris 100 mM, Boric Acid 9 mM, EDTA 1 mM) at room temperature (18°C) and constant 200 V for 3 hours. Polyacrylamide gels were stained with silver nitrate according to the protocol described by Herring *et al.* (1982).

#### **Statistical Analysis**

The allelic and genotypic frequencies, expected means, observed and expected Nei's heterozygosities (HE=1- $\Sigma P_i^2$ , where  $P_i$  is the frequency of allele i) and Hardy-Weinberg equilibrium were calculated using PopGene32 program, ver 1.31, Canada (Yeh *et al.*, 1997).

#### RESULTS

#### PCR-SSCP Analysis of CAST Gene

The amplification of a 622 bp fragment of the *CAST* exon 1 gene was successful in our first attempt. All extracted DNAs from sheep blood samples yielded a specific single band PCR product without any nonspecific band. Therefore, the PCR products were directly used for SSCP analysis.

The allelic variation in the CAST gene was by PCR-SSCP. The nonexamined denaturing gel electrophoresis enabled visualization of ssDNA and analyzed for SSCP band patterns. In this study a total of four SSCP patterns were observed in the examined sheep (Figure 1). The frequencies of the observed genotypes were 0.31, 0.04, 0.63 and 0.02 for AA, BB, AB and AC, respectively. Allele frequencies were 0.63, 0.36 and 0.01 for A, B and C respectively (Table 1).

Statistically estimated parameters for *CAST* locus in Makoei sheep have been presented in table 2. The chi-square test showed significant (P < 0.01) deviation from Hardy-Weinberg equilibrium for this locus in the studied population.

#### DISCUSSION

In the present study, three alleles (A, B, and C) and four genotypes (AA, AB, BB, and AC) were observed for *CAST* gene in "Makoei" sheep breed in West Azerbaijan, Iran. The most frequent allele and genotype in the "Makoei" sheep breed were 63.13% and 31% for allele *A* and genotype *AB*, respectively. The results obtained from this study revealed the polymorphism in the

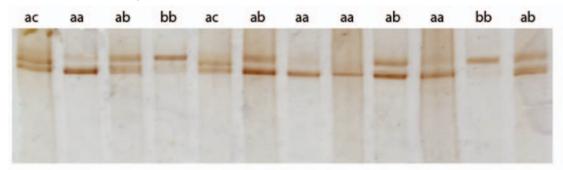


Figure 1. SSCP polymorphism of Makoei sheep *CAST* gene. Four different PCR-SSCP patterns (genotype) were identified.

Table 1. Observed allele and genotypic frequencies for CAST locus in Makoei sheep.

Α	В	С	AA	BB	AB	AC
0.6313	0.3586	0.0101	0.31	0.04	0.63	0.02

Exp-Het	Exp-Hom	Het (Nei)	Ave-Het	Obs-Hom	Obs-Het				
0.4752	0.5248	0.4728	0.4728	0.3434	0.6566				

Table 2. Statistically estimated parameters for CAST locus in Makoei sheep

*CAST* gene of Makoie sheep. Variation in non-coding and coding regions of the ovine *CAST* gene has been reported by several researchers (Palmer *et al.*, 1998; Palmer *et al.*, 2000; Roberts *et al.*, 1996; Zhou *et al.*, 2007).

Polymorphism study on the same region of the CAST gene in Kurdi sheep by PCR-SSCP revealed three genotypes including aa, ab and ac (Nassiry et al., 2006). The polymorphism in the exon 1 of the CAST in sheep was also reported by other researchers using PCR-RFLP technique (Gabor et al., 2009; Mohammadi et al., 2008; Palmer et al., 1998). In goats and bovine the exon 6 of CAST gene were investigated for polymorphisms and a number of allelic variants were identified in these species (Zhou and Hickford 2008; Zhou et al., 2007). Fortest (2007) reported higher frequencies of CAST gene's allele A compared to the allele B in Nellore (0.66), Rubia Gallega (0.72), Canchim (0.62), Brangus (0.78) and Pardo Suico (0.80)cattle.

There are several studies on the association of *CAST* gene polymorphism and meat quality in animals. Schenkel *et al.* (2006) reported a significant association between allele *C* of bovine *CAST* gene and meat tenderness. Kuryl *et al.* (2003) reported that *CAST* gene may be considered as a candidate gene for pig carcass quality. Association between allele *D* and *F* of porcine *CAST* gene and meat quality traits was also reported by Kapelański *et al.* (2004).

Palmer *et al.* (1999) found allelic frequencies of 0.69 and 0.70 for allele *A* in Dorset Down and Coopworth, respectively, which was in close agreement with the frequency of the allele *A* in Makoei sheep in the present study. In contrast, they reported

that frequencies of alleles A and B in Corriedale and Ruakura were 0.27 and 0.41, respectively. Different frequencies for the alleles of the CAST gene have been reported in Iranian Baluchi sheep with 0.70 for allele A, 0.08 for allele B, and 0.22 for allele C. Genotypes BC and CC, which presented, respectively, the 0.03 and 0.04 frequencies in Baluchi sheep, were not observed in Makoei sheep (Tahmoorespur et al., 2007). Two allelic systems of polymorphic variants (M and N) in the region of ovine CAST locus have been described by PCR-RFLP method (Palmer et al., 1998; Shahroodi et al., 2005). According to Palmer et al. (1998), allelic frequencies were 77% and 12% for the Mand N in Corriedale sheep, respectively.

The present study was the first attempt for identification of *CAST* gene variation in Iranian Makoei sheep. Further studies are required to investigate the relationship between *CAST* gene polymorphisms and the performance traits in Makoei sheep.

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#### REFERENCES

 Casas, E., White, S. N., Wheeler, T. L., Shackelford, S. D., Koohmaraie, M., Riley, D. G., Chase, C. C., Jr., Johnson, D. D. and Smith, T. P. 2006. Effects of Calpastatin and Micro-calpain Markers in Beef Cattle on Tenderness Traits. J. Anim. Sci., 84: 520-525.

- Chung, H. Y., Davis, M. E. and Hines, H. C. 1999. A DNA Polymorphism of the Bovine Calpastatin Gene Detected by SSCP Analysis. *Anim. Genet.*, **30**: 80.
- Ciobanu, D. C., Bastiaansen, J. W., Lonergan, S. M., Thomsen, H., Dekkers, J. C., Plastow, G. S. and Rothschild, M. F. 2004. New Alleles in Calpastatin Gene Are Associated with Meat Quality Traits in Pigs. J. Anim. Sci., 82: 2829-2839.
- 4. Fortest, T. M. R. S. 2007. Polymorphism in *CAPN1, CAST, TG* and *DGTA1* Genes as Possible Markers for Bovine Meat Quality Traits in Zebu and Crosses Slaughtered in Young Age. Animal Reproduction, University of Sao Paulo, Brazil.
- Gabor, M., Trakovicka, A. and Miluchova, M. 2009. Analysis of Polymorphism of CAST Gene and CLPG Gene in Sheep by PCR-RFLP Method. Sci. Pap. Anim. Sci. Biotechnol., (Lucrări ştiințifice Zootehnie şi Biotehnologii) 42: 470-476.
- Herring, A. J., Inglis, N. F., Ojeh, C. K., Snodgrass, D. R. and Menzies, J. D. 1982. Rapid Diagnosis of Rotavirus Infection by Direct Detection of Viral Nucleic Acid in Silver-Stained Polyacrylamide Gels. J. Clin. Microbiol., 16: 473-477.
- Kapelański, W., Grajewska, S., Kurył, J., Bocian, M., Jankowiak., H. and Wiśniewska, J. 2004. Calpastatin (CAST) Gene Polymorphism and Selected Meat Quality Traits in Pigs. *Anim. Sci.*, 22: 435-411.
- Koohmaraie, M. 1992. The Role of Ca(2+)dependent Proteases (calpains) in Post Mortem Proteolysis and Meat Tenderness. *Biochimie*, 74: 239-245.
- Koohmaraie, M. 1996. Biochemical Factors Regulating the Toughening and Tenderization Processes of Meat. *Meat Sci.*, 43: 193-201.
- Krzecio, E., Kocwin-Podsiadla, M., Kuryl, J., Zybert, A., Sieczkowska, H. and Antosik, K. 2008. The Effect of Interaction between Genotype CAST/Rsai (Calpastatin) and MYOG/Mspi (Myogenin) on Carcass and Meat Quality in Pigs Free of RYR1T Allele. Meat Sci., 80: 1106-1115.
- Kuryl, J., Kapelański, W., Pierzchała, M., Grajewska, S. and Bocian, M. 2003. Preliminary Observations on the Effect of Calpastatin Gene (CAST) Polymorphism on Carcass Traits in Pigs. *Anim. Sci. Pap. Rep.*, 21: 87-95.

- Lonergan, S. M., Ernst, C. W., Bishop, M. D., Calkins, C. R. and Koohmaraie, M. 1995. Relationship of Restriction Fragment Length Polymorphisms (RFLP) at the Bovine Calpastatin Locus to Calpastatin Activity and Meat Tenderness. J. Anim. Sci., 73: 3608-3612.
- Mohammadi, M., Beigi Nasiri, M. T., Alami-Saeid, K. h., Fayazi, J., Mamoee, M. and Sadr, A. S. 2008. Polymorphism of Calpastatin Gene in Arabic Sheep Using PCR-RFLP. *Afr. J. Biotechnol.*, 7: 2682-2684.
- Nassiry, M. R., Tahmoorespour, M., Javadmanesh, A., Soltani, M. and Foroutani Far, S. 2006. Calpastatin Polymorphism and Its Association with Daily Gain in Kurdi Sheep. *Iran. J. Biotechnol.*, 4: 188-192.
- 15. Palmer, B. R., Robert, N. and Kent, M. P. 1999. A Candidate Gene Approach to Animal Quality Traits. *Proc. New Zealand Soc Anim. Prod.*, **57**: 294-296.
- Palmer, B. R., Roberts, N., Hickford, J. G. and Bickerstaffe, R. 1998. Rapid Communication: PCR-RFLP for Mspi And Ncoi in the Ovine Calpastatin Gene. J. Anim. Sci., 76: 1499-1500.
- 17. Palmer, B. R., Su, H. Y., Roberts, N., Hickford, J. G. and Bickerstaffe, R. 2000. Single Nucleotide Polymorphisms in an Intron of the Ovine Calpastatin Gene. *Anim. Biotechnol.*, **11**: 63-67.
- Pipalia, D. L., Joshi, C. G., Rank, D. N., Brahmkshtri, B. P. and Solanki, J. V. 2004. PCR-SSCP Typing Of MHC in Cattle and Buffaloes. *Ind. J. Anim. Sci.*, **74**: 637-639.
- 19. Roberts, N., Palmer, B., Hickford, J. G. and Bickerstaffe, R. 1996. PCR-SSCP in the Ovine Calpastatin Gene. *Anim. Genet.*, **27**: 211.
- 20. Saadat-Noori, M. and Siah-Mansoor, S. 1992. *Sheep Husbandary and Management*. Ashrafi Publication, Tehran, PP.135-136.
- Schenkel, F. S., Miller, S. P., Jiang, Z., Mandell, I. B., Ye, X., Li, H. and Wilton, J. W. 2006. Association of a Single Nucleotide Polymorphism in the Calpastatin Gene with Carcass and Meat Quality Traits of Beef Cattle. J. Anim. Sci., 84: 291-299.
- Seiler, J. 1994. The Future Role of Molecular Genetics in the Control of Meat Production and Meat Quality. *Meat Sci.*, 36: 29.
- 23. Shahroodi, F. E., Nassiry, M. R., Valizadeh, R., Nosrati, M., Javadmanesh, A. and

Tahmourespour, M. 2005. The Genetic Polymorphism of Calpastatin Gene in Karakul Sheep. J. Agric. Sci. Nat. Reso. Khazar, **2**: 1-10.

- Tahmoorespur, M., Valizadeh, R., Shahroodi, F. E., Nassiry, M. R., and Sharif, A. 2007. Study of Calpain Gene Polymorphism and Its Association to Daily Gain in Baluchi Sheep. *Agric. Sci. Technol. J.*, **20**: 146-153.
- Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M. and Goll, D. E. 1995. Is Z-disk Degradation Responsible for Postmortem Tenderization? *J. Anim. Sci.*, 73: 1351-1367.
- 26. Yeh, F. C., Yang, R. C., Boyle, T. B. J., Ye, Z. H. and Mao, J. X. 1997. POPGENE, the User-Friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.
- Zhou, H. and Hickford, J. G. 2008. Allelic Variation of the Bovine Calpastatin (CAST) Gene. *Mol. Cell. Probes*, 22: 129-130.
- Zhou, H., Hickford, J. G. and Gong, H. 2007. Polymorphism of the Ovine Calpastatin Gene. *Mol. Cell. Probes*, 21: 242-244.

## شناسایی چندشکلی های ژن کالپاستاتین در گوسفند ماکویی با استفاده از تکنیک PCR-SSCP

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چکیدہ

کالپاستاتین (CAST) یک مهارکننده اختصاصی کالپین ها می باشد که در تردی و ساختن سلولهای عضلانی نقش دارد. در مطالعه حاضر چند شکلی CAST در گوسفندان نژاد ماکویی با استفاده از تکنیک PCR-SSCP مورد ارزیابی قرار گرفت. DNA ژنومی از نمونه های خون کامل تعداد ۱۰ رأس گوسفند ماکویی تهیه گردید. قطعهای به اندازه ۶۲۲ جفت باز از ناحیه اگزون یک با بکارگیری آغاز گرهای اختصاصی لو کوس CAST تکثیر شد. با استفاده از روش SSCP ، محصولات PCR ژن *CAST بر رو*ی ژل غیر دناچوره کننده الکتروفورز شدند. چهار الگوی SSCP که بیانگر چهار ژنوتیپ مختلف بودند شناسایی گردید. فراوانی ژنوتیپهای مشاهده شده AA ، BB هر AA به ترتیب عبارت بودند از: ۳۱،۰۰، ۲۰٬۰۰ و ۲۰/۰ .فراوانی آللی برای آللهای A، Bو C به ترتیب عبارت بودند از: ۵۹/۰۰، ۲۵۸۶ و ۱۰/۰ .مقدار هتروزیگوسیتی مشاهده شده (Obs-Het) ژن *CAST بر رای رای رای رای رای الحا*ی رای آللی برای آللهای A، G *CAST در گروس CAST در در او ان در ای تو تیپهای مشاهده شده AA با کا* و Cos