Calpastatin polymorphism and its association with daily gain in Kurdi sheep

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Abstract
Association of genetic polymorphism in the calpastatin (*CAST*) gene with average daily gain was examined in Iranian purebred Kurdi sheep. The genotypes for *CAST* were determined by the PCR-SSCP method. Blood samples were collected from 84 purebred Kurdi sheep belonging to the Kurdi Breeding Station located in the Khorasan province, north-east of Iran. Extraction of genomic DNA was based on the Guanidinium Thiocyanate-Silica gel method. Three genotypes including *aa*, *ab* and *ac* with frequencies of 0.55, 0.32 and 0.13, were observed in this population. Chi-square test confirmed Hardy-Weinberg equilibrium for the *CAST* loci. Average heterozygosity (37%) of the *CAST* locus for Kurdi sheep was slightly low. Daily gain birth to weaning (GBW); weaning to six months (GWS); six to nine months (GSN) and nine to yearling (GNY) were analyzed by a statistical model comprising SSCP (Single strand conformation polymorphism) and significant effect (P<0.05) of *CAST* genotypes was observed for GBW only.
Keywords: Calpastatin; Polymorphism; Kurdi Sheep; daily gain.

INTRODUCTION

Genetic polymorphism in native breeds is a major concern considering the necessity of preserving genetic resources. It is very important to characterize genetically indigenous breeds (Bastos *et al*., 2001). Marker-assisted selection is one of the new DNA based methods that improves accuracy and progress of selection in animal programmes. Calpastatin (*CAST*) gene is located on the fifth chromosome of sheep and plays important roles in formation of muscles, degradation and meat tenderness after slaughter. Calpastatin and calpain deserve special attention because of their major role in meat production. The calpain-calpastatin system (CCS) comprises a family of calcium dependent neutral proteinases, with CAST acting as a specific inhibitor of μ and m-calpain proteases. The CCS is found in most animal tissues and influences many important processes including muscle development and degradation, postmortem meat tenderization, cataract formation and fertility (Merin *et al*., 1998). A high degree of polymorphism at the *CAST* locus has also been reported in studies with Dorset Down, Dorset Down × Coopworth, Corriedale sheep (Palmer *et al*., 1999a), Angus bulls (Chung *et al*., 2001), crossbred steer and pigs (Kurly *et al*., 2003). By using a molecular genetic approach to study meat quality in sheep, Palmer *et al*. (1999b) have chosen the ovine *CAST* gene as a candidate gene for meat quality. Palmer *et al*. (1998) have described a two allele systems of polymorphic variants (M and N) in a region of the ovine *CAST* by the PCR-RFLP method. Also, Palmer *et al*. (1998), Chung *et al*. (2001) and Tahmoorespour (2005) have described a three allele systems of polymorphic variants (*CAST* a, b, and c) by PCR-SSCP in a region of the ovine and cattle *CAST*. Since 1998, Palmer *et al*. (1999a) have carried out slaughter trials on small groups of Dorset Down and Dorset Down × Coopworth lambs to ascertain any association between weight gain and meat quality traits with the alleles at the *CAST* locus. In previous studies, sheep with the *ac* genotype (in the PCR-SSCP method) have been shown with increased live weight gain (+12-17%, P<0.05), increased age-corrected carcass weight (+15-18%, P<0.05), but increased Longissimus dorsi shear force (+4-12%, no significant) compared to sheep with the *AA* genotype. Kurly *et al*. (2003) observed that Pigs with the genotype *DD*

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at locus *CAST*/*Msp*I and FF at locus *CAST*/*Rsa*I had less fatty acid, thinner back fat and a lower weight of back fat with skin. These observations suggest that calpastatin may be considered as a candidate gene for gain and lean content of carcass in cattle and sheep.

In this study we have chosen *CAST* as a candidate gene for daily gain in sheep because of the evidence in cattle (Chung *et al*., 2001), pig (Kurly *et al*., 2003) and sheep (Palmer *et al*., 1999a; Tahmoorespour *et al*., 2005) implicating a role for the *CAST* gene in skeletal muscle (Edyta *et al*., 2002). The aim of this study was to determine existence of any association of polymorphism at the *CAST* locus with gain characteristics of the Kurdi sheep breed in the Shirvan Breeding Station.

MATERIALS AND METHODS

Animals and DNA extraction: Blood samples were randomly collected from 84 purebred Kurdi sheep belonging to the Kurdi Breeding Station located in Shirvan, Northern Khorasan Province, Iran. DNA was extracted from 100 µl of blood as described by Boom *et al*. (1990).

PCR: Reaction was carried out in a total volume of 25 µl which consisted of 50-100 ng of template DNA, 2.5 ul PCR buffer 10X (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 10 pM of each forward and reverse primers and 1 U of *Taq* DNA polymerase.

Thermal conditions started with a primary denaturation at 95°C (3 min) followed by thirty-five cycles at 95°C (1 min), 62°C (1 min), and 72°C (2 min) followed by 72°C (8 min) for the final extension. Exon 1C/1D from domain 1 region including the intron of the ovine *CAST* gene were amplified to a 622 bp fragment using primers based on the sequences of *CAST* gene. Primer sequences were:
ovine 1C: 5′-TGGGGCCCAATGACGCCATCGATG-3′
ovine 1D: 5′-GGTGGAGCAGCACTTCTGATCACC-3′
PCR products were visualized after electrophoresis on 1.5% agarose gel stained with etithium bromide.

SSCP: For genotyping of the *CAST* locus, PCR products were diluted with 12 µl of running buffer that included: 80% formamid, bromophenol blue 1%, xylenecyanol 1%, 0.5 M EDTA and 10 M NaOH. After incubation at 95°C for five minutes, they were immediately placed on ice the genotypes were then detected using 8% non-denaturing polyacrylamide gel containing 10% glycerol. The mixture was electrophoresed for 3-4 h at 250 V at 10°C. DNA fragments were visualized using the silver staining method.

Statistical analysis: The allelic and genotypic frequencies, expected mean, observed and Nei's heterozygosities (H_E=1-Σp^2_i, where p_i is the frequency of allele i) and Hardy-Weinberg equilibrium test were calculated using PopGene32 (ver 1.31) program [http://cc.oulu.fi/~jaspi/popgen/popdown.htm].

Only 75 sample were used for statistical analysis. Average daily gain from birth to weaning (GBW), weaning to six month (GWS), six to nine month (GSN) and from nine month to yearling age (GNY) were analyzed using the mixed model by JMP software (version 4.01; SAS Institute Inc, NC. USA) with the following statistical model:

$$Y_{ijklm}=\mu+S_i+B_j+G_k+G*S_{ki}+Sire_l+e_{ijklm}$$

Where:
Y_{ijklm}= mean value of the trait;
μ= general mean;
S_i= effect of sex (i=1 and 2)
B_j= effect of birth type (j= 1 and 2)
G_k= effect of genotype (k= 1, 2 and 3)
$Sire_l$= effect of sire (l= 1, 2, …26)
$G*S_{ki}$ = the interaction between sex and genotype
e_{ijklm}= random error

The non-significant effects and corresponding interactions were discarded for the final analysis. Least square means (LSM) were compared using the Duncan difference test (JMP ver. 4.01) with a comparison error rate of 0.05.

RESULTS

Genetic variability: Three genotypes namely *aa*, *ab* and *ac* were detected in this population. The frequencies of the *CAST* genotypes, alleles and χ^2 test are shown in Table 1. Genotypes *aa*, *ab* and *ac* were observed at frequencies of 54.76, 32.14% and 13.09%,

Table 1. Observed Allele and genotypic frequencies and χ^2 test for *CAST* locus.

Locus	a	b	c	aa	ab	ac	χ^2
CAST	0.78	0.16	0.06	0.55	0.32	0.13	6.96^{ns}

ns: non-significant

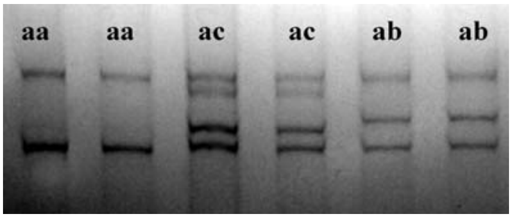

Figure 1. SSCP polymorphism within ovine *CAST* gene. Three different patterns (genotype).

respectively. The genotypes of bb, bc, and cc were not observed in Iranian Kurdi sheep. The Chi-Square (χ^2) test confirmed the Hardy-Weinberg equilibrium in this population (P<0.05). Allele frequencies for *a*, *b*, and *c* were 0.78, 0.16, and 0.06, respectively. Figure 1 shows the electrophoresis of *CAST* genotypes after SSCP. Observed heterozygosity (45%), expected heterozygosity (37%), Nei's heterozygosity (37%) and average heterozygosity (37%) of the *CAST* locus for Kurdi sheep of the Shirvan Breeding Station were low (Table 2).

Table 2. Observed, expected and average heterozygosities of *CAST* locus.

Locus	Obs-Het	Exp-Het*	Nei**	Ave-Het
CAST	0.46	0.37	0.37	0.37

* Expected heterozygosity were computed using Levene (1949).
** Nei's (1973) expected heterozygosityl.

Association analysis: The analysis of variance (ANOVA) for GBW, GWS, GSN and GNY is summarized in Table 3. Significant differences (P<0.01) in the GBW trait were observed for the *CAST* genotypes. For the GNY, *CAST* genotypes contributed significantly to the variation at the lower probability level (P<0.10). Also, the genotype effect for GWS and GSN were not significant. Least square means and standard errors (SE) for GBW, GWS, GSN and GNY traits for each relevant *CAST* genotype are shown in Table 4. The ab genotype was associated with higher average daily gain than aa and ac in Iranian Kurdi sheep. The least square means of the ab genotype (215.22 g) were significantly different (P<0.05) from that of the aa (204.88 g) and ac (172.62 g). For GNY, ab genotype was associated with a higher gain than aa and ac genotypes (P≤0.10). The present data did not show any influence of *CAST* genotypes on the other studied traits. Significant differences (P<0.01) in the GBW trait were observed for sex, birth type, sire effects and genotype×sex interaction.

DISCUSSION

Genetic variability: We observed only three alleles (*a*, *b*, and *c*) and three genotypes (*aa*, *ab*, and *ac*) in the Kurdi sheep breed in Shirvan. The most frequent allele and genotype in the Kurdi breed were 0.77 and 54.76% for allele a and genotype aa, respectively. Palmer *et al*. (1999b) found allelic frequencies of 0.69 and 0.70 for allele *a* in Dorset Down and Coopworth, respectively, which is in agreement with these results. In contrast, they reported that frequencies of alleles *a* and *b* in Corriedale and Ruakura were 0.27 and 0.41, respectively, while in Kurdi sheep allele *c* was presented at the frequency of 0.06. Tahmoorespour (2005) found allelic frequency of 0.70 for allele *a*, 0.08 for allele *b*

Table 3. ANOVA for GBW, GWS, GSN and GNY.

SOV	df	MS (GBW)	MS (GWS)	MS (GSN)	MS (GNY)
Sex	1	8568.60***	2832.09	15636.61	16445.97***
Sire	26	656.53**	1359.40	1139.04**	590.14
Birth type	1	3766.05***	409.60	204.78	2195.73*
Genotype	2	2956.78***	1050.63	652.09	1862.48*
Genotype × Sex	2	931.81*	16.79	2856.00**	1480.08
Error	42	316.52	1639.15	573.70	699.62

*** P<0.01; ** P<0.05; * P<0.10
a-c: values in the same column lacking a common superscript differ (P<0.05).

Table 4. Least square means (LSM) and standard error (SE) for GBW, GWS, GSN and GNY (g/day) by *CAST* genotypes.

	GBW		GWS		GSN		GNY	
	LSM	SE	LSM	SE	LSM	SE	LSM	SE
aa	204.88 ^{a}	4.76	85.20 ^{a}	10.82	13.10 ^{a}	6.40	65.06 ^{a}	7.56
ab	215.22 ^{b}	5.75	76.62 ^{a}	13.09	25.57 ^{a}	7.74	85.55 ^{b}	8.56
ac	172.62 ^{c}	7.90	101.38 ^{a}	17.99	20.74 ^{a}	10.64	63.58 ^{a}	11.76

a-c: values in the same column lacking a common superscript differ (P<0.05).

and 0.22 for allele *c* in Baluchi sheep. Similar number of homozygous individuals was observed in the progeny of Dorset Down × Coopworth sheep (Palmer *et al*., 1998). The *ab* genotype was not found in Dorset Down × Coopworth lambs. Genotypes *bc* and *cc*, which had frequencies of 0.03 and 0.04 respectively, in Baluchi sheep (Tahmoorespour 2005), were not observed in Kurdi sheep. Palmer *et al*. (1998), Eftekhari Shahroudi *et al.* (2005) and Elyasi *et al.* (2005) have described a two alleles system of polymorphic variants (*M* and *N*) in a region of the ovine *CAST* locus by PCR-RFLP method. Elyasi *et al.* (2005) reported an allele frequency of 69%, 48% and 50% for the *M* allele in Ghezel, Arkhar Merino and Ghezel × Arkhar Merino breeds, respectively. According to Palmer *et al*. (1998), allelic frequencies were 77% and 12% for the alleles *M* and *N* in Corriedale sheep, respectivly. A high degree of polymorphism at the *CAST* locus has also been reported in studies with Angus calves and pigs. In Angus calves, observed genotypes were *AA*, *AB*, and *AB* (*A* and *B* alleles) for the *CAST* (exon 1C/1D) locus with the PCR-SSCP method. The frequencies for these alleles were 0.31 for *A* and 0.69 for *B* allele (Chung *et al*., 1999).

Kurdi sheep showed a low degree of genetic variability for the *CAST* locus, which may be explained by the conservation and breeding method that has been carried out. In this station, only a few rams have been used for breeding. With respect to a low number of the effective population, the inbreeding rate is high and so heterozygosity and genetic variability is low. Therfore, using rams from other stations is recommended to solve the increasing inbreeding problem in that flock. Although we observed low variability for this locus, on the other hand, these data provide evidence that the Kurdi sheep breed is polymorphic for *Calpastatin* locus, which opens interesting prospects for future selection programs, especially marker-assisted selection for gain and meat quality traits.

Calculated heterozygosity at the *CAST* locus in this Station was low as a result of the closed breeding system which, therefore caused an increase in χ^2 value.

Association analysis: This study showed a genetic association between genotype *ab* and average daily gain from birth to weaning. Significant differences ($P<0.10$) in the GBW trait were observed for the *CAST* genotypes. Similarly, Tahmoorespour (2005) observed significant differences ($P<0.10$) between the *CAST* genotypes for GBW in Baluchi sheep. Significant effect ($P<0.05$) of the interactions between genotype and sex were verified for the GBW. Significant association of the genotypes with GSN was not observed, nevertheless, lambs with the *ab* genotypes had higher gain than those with *aa* genotypes. A significant difference ($P<0.10$) among the genotypes for GNY was observed. Animals with the *ab* genotype for *CAST* calpastatin had higher GBW ($P<0.05$) than aa and ac genotypes. The results of this study showed that lambs with the *ab* genotype produce 10.34 g/d and 42.60 g/d more gain compared with the *aa* and *ac* genotypes, respectively. Similarly, Tahmoorespour (2005) reported that in Baluchi sheep the *ab* (190.2 g) had significantly ($P<0.05$) higher GBW than *ac* (185.5 g) and *aa* (182.4 g) genotypes. In contrast to our results, Palmer *et al*. (1999a) compared the association of *aa*, *ab* and *ac* genotypes at the *CAST* locus in Dorset Down and Dorset Down × Coopworth lambs with weight gain. They had indicated an association of the *CAST ac* genotype with increased live weight gain (+12-17%, $P<0.05$) compared to *aa* genotypes. Also, there appeared to be little difference in growth or quality traits between lambs with *aa* and *ab* genotypes. It seems that further studies on this subject in sheep are required. Association of the *CAST* gene types has also been reported in cattle and pig. For example, Chung *et al*. (2001) studied association between PCR-SSCP genotypes (*AA*, *AB* and *BB*) and weight at day 56 (W56) and average daily gain (ADG) in two hundred and thirty three purebred Angus calves. They reported that *AB* and *BB* genotypes had higher weight at day 56 ($P< 0.05$) than that of the *AA* genotype. Also, they did not find any significant association between *CAST* genotypes and average daily gain. Kocwin-podsiadla and Kurryl (2003) were observed a relationship between the *CAST/Msp*I genotype and yield of loin ($P< 0.05$). There has been simultaneous evaluation of the effect *CAST* gene upon in meat quality and tenderization. Casas *et al*. (2006) observed that the meat of cattle inheriting the *TT* genotype at the *CAST* locus had meat that was more tender than those inheriting the *CC* genotype. Schenkel *et al*. (2006) showed that the *CAST* SNP was associated with shear force across days of postmortem aging ($P= 0.05$) and genotype *CC* yielded beef that was more tender than that of *GG*. They also indicated that genotype *CC* had a greater fat yield (1.44±0.56 %; $P= 0.037$) than *GG* genotype.

This was the first study using polymorphism of the *CAST* locus to understand genetic variability of Kurdi sheep in Iran. Very little information is currently available to compare different Iranian sheep breeds. The present study may be regarded as the beginning of attempts to understand the genetic variability of native sheep breeds in the Khorasan region. It can be concluded that although CAST polymorphism is associated with average daily gain from birth to weaning GBW and nine month to yearling GNY at the Shirvan Breeding Station, but further analysis needs to be conducted on the association between daily gain and *CAST* genotypes.

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