VARIATION IN EXON 10 OF THE OVINE CALPAIN3 GENE AND ITS ASSOCIATION WITH GROWTH AND CARCASS TRAITS IN EGYPTIAN BARKI LAMBS

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Growth and carcass traits are most economically important traits in sheep. These traits are quantitative and characterized by a continuous variation of phenotype values. The phenotype of an animal for a quantitative trait depends on its genotype at several loci called quantitative trait loci (QTL), as well as environmental factors (Van Laere, 2005).

There are two basic methods of QTL identification: 1) the candidate gene approach, 2) the genome scan approach. The candidate gene approach is a powerful method for identifying the QTL responsible for genetic variation in the traits of interest in agriculture animal species (Rothschild and Soller, 1997). However, the candidate gene approach is limited by the biological information available for the traits of interest.

In sheep, skeletal muscle constitute about 28-30% of body mass. The rate and extent of skeletal muscle growth depend mainly on three factors: 1) the rate of muscle protein synthesis, 2) the rate of muscle protein degradation, 3) the number and size of skeletal muscle cells (Lee *et al*., 2001). Studies on the rate of muscle growth in livestock animals have shown that the rate of muscle growth are mainly determined by changes in the rate of muscle protein degradation, with little or no change in the rate of muscle protein synthesis (Goll *et al*., 1989).

In mammals, calpain system which is involved in skeletal muscle plays an important role in formation and degradation of muscle protein and postmortem meat tenderization (Merin *et al*., 1998). This system has three isoforms of calpains, two are calcium dependant; calpain 1 (CAPN1) and calpain 2 (CAPN2) and the third calpain 3 (CAPN3), is independent of calcium. The calcium dependant calpains are further regulated by calpastatin, which inhibits the activity of calpains. A number of studies have shown that this system has an important role both in the muscle growth and muscle wasting that occurs in various muscular dystrophies and other conditions accompanied by loss of muscle mass and in metabolic and turnover of muscle proteins. Both CAPN1 and CAPN2 are primarily related to muscular dystrophy and growth, and play a crucial role in the differentiation of pre-adipocyte to adipocyte (Patel and Lane, 1999) and myofibrillar organization (Poussard *et al*., 1996). Also,

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it is specifically linked to connective muscle in regions where proteolysis has been associated with post-mortem meat tenderness (Tayler *et al*., 1995). CAPN3 participates in the regulation of myogenesis. Mutation in CAPN3 result in an autosomal recessive and progressive form of limb-girdle muscular dystrophy called limb-girdle muscular dystrophy type 2A (Beckmann and Spencer, 2008).

CAPN3 protease is encoded by CAPN3 gene which located on the *Ovis aries* chromosome 7. The full extent of variation in Ovine CAPN3 gene has not been characterized. Zhou *et al*. (2007) cited that the most variable region appear to be in exon 10 with three allelic polymorphisms. Fang *et al*. (2013) detected four allelic polymorphisms in the same region.

A few number of studies evaluated the association between CAPN3 gene variation and growth and carcass traits of farm animal.

In sheep, a single nucleotide polymorphism (SNP) in intron 11 of CAPN3 gene has been associated with birth weight (Chung *et al*., 2007), and the yield of retail lamb meat cuts (Bickerstaffe *et al*., 2008). The allelic variants, which detected in exon 10 by Fang *et al*. (2013), were found to be associated with shoulder weight and hot carcass weight ($P< 0.05$).

In cattle, variation in 5′- untranslated region (5′-UTR) was associated ($P< 0.05$) with carcass weight (Hou *et al*., 2010). No associations were found between the variation in genomic sequence of CAPN3 gene and growth and carcass traits of Brahman calves (Café *et al*., 2010). Two SNPs in CAPN3 gene (rs 109806627 & rs 136324366) were found to be associated ($P< 0.05$) with weaning weight of Angus calves (Bailey, 2010).

In chickens, variation in CAPN3 gene has been associated with body weight, carcass weight, breast weight and leg muscle weight (Zhang *et al*., 2009). The SNP (g. 15486 C>T) which detected by Felicio *et al*. (2013) has been associated ($P<0.05$) with carcass weight.

From the previous, the CAPN3 gene is considered as a candidate gene affecting growth and carcass traits of Egyptian Barki sheep.

The objective of this study was to detect the allelic variation in exon 10 of CAPN3 gene and its association with growth and carcass traits in Egyptian Barki sheep.

MATERIALS & METHODS

Data collection

This study was carried out at Maryout Research Station, which is one of the experimental stations of Desert Research Center. Twenty four ram males of Barki sheep that born at the same week, phenotyped for growth traits (birth weight, weaning weight, post-weaning daily gain and marketing weight).

Blood samples were drawn from the jugular vein into 5 ml heparinized tubes. These samples were stored at -80°C for thirty months, whereupon genomic DNA extracted with the use of DNA extraction kit (Maxwell® RSA Blood DNA Kit, Promega, CAT# AS1400).

At marketing age (9 months), 24 male lambs were fasted for 18 hours and then weighted to record the fasted body weight. Slaughtering was carried out by severing the carotid artery and jugular veins. After slaughter and bleeding, carcasses were skinned and eviscerated before weighing. Weights of all abdominal and thoracic offals (trachea, lungs, heart, liver, testes, spleen, kidneys, abdominal fat and kidney fat) were recorded immediately after removal from the body. The rumen and reticulum were cleaned and washed under cold running water, and then they were weighed. Empty body weights were recorded post-slaughter, and then all carcasses were cooled at an average temperature of 4°C for 24 h to evaluate cold carcass weight (Field *et al*., 1963).

After cooling, each carcass was divided into seven cuts (Legs, Loins, Racks, Flank, Shoulders, Neck and Tail) according to the Egyptian wholesale mutton cuts as described by Hamada (1976). Cold carcasses and wholesale cut were weighed to calculate percentages of cold carcass weight. The 9-10-11 rib cut was separated into its physical components (lean meat, fat and bone), which were expressed as percentages of the weight of the whole rib cut.

Polymerase chain reaction

Two specific primers were used to amplify 168 pb from exon 10 of the ovine CAPN3 gene. The sequence of these two primers based on the reference of Fang et al. (2013), and was as follow: Forward: 5′-CTCTCAGGATGTCCTACG-3′ and Reverse: 5′-CTGGGAAGTTGCGGCAG-3′.

PCR carried out in a total reaction volume of 20 ul, containing 2.5 ul of 10x PCR buffer. 1.5 mM of $MgCl_2$, 150 *u*M of dNTP (Eppendorf, Hamburg, Germany), 0.25 uM of each primer, 50 ng of genomic DNA and 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany). The amplification conditions were as follows: initial duration at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. A final 5 min extension step was completed at 72°C.

SSCP analysis

A 1.5 ul of each PCR product was mixed with 13.5 ul of loading dye containing 98% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylenecyanol. After denaturation at 105°C for 6 min, samples were cooled on wet ice and loaded onto 14% polyacrylamide gel. PCR products representative of the first three known CAPN3 alleles which detected by Fang *et al*. (2013), were included in the polyacrylamide gel (well number 3),

and their banding patterns were used as standards for determining the alleles present in individual sheep. This gel was electrophoresed at 300 V for 18 h at 5°C in 0.5x TBE buffer, followed by silver staining as described by Sanguinette *et al.* (1994).

Statistical analysis

Allelic and genotypic frequencies of CAPN3 gene in exon 10 were calculated using simple gene counting method (Falconer and Mackey, 1996).

$$p = \frac{AA + 1/2(AB + AC)}{N}$$

$$q = \frac{BB + 1/2(AB + BC)}{N}$$

$$r = \frac{CC + 1/2(AC + BC)}{N}$$

Where P = frequency of A allele, q = frequency of B allele, r = frequency of C allele and N = the number of genotyped animals.

Hardey-Weinberg equilibrium was tested by comparing expected and observed genotypic frequencies using χ^2 test by this formula: $\chi^2 = \sum \frac{(O-E)^2}{E}$

Where: O = the observed genotypic frequency and E = the expected genotypic frequency.

Associations of CAPN3 genotype with growth and carcass traits were determined by analysis of variance of quantitative traits. General Linear Mixed Model (GLMM) procedure in SAS (1989) was used to perform the analysis. Fixed effect of CAPN3 genotype and random effect of sire were included as independent variables in the model. Age at weaning was included in the model as a co-variate.

$$Y_{ijK} = \mu + G_i + S_j + e_{ijk}$$

Where: Y_{ijK} = weight of the component Y; μ = overall mean, G_i = fixed effect of CAPN3 genotype, S_j = random effect of sire; and eij_K = the random error assumed N.I.D. (0, σ^2, e).

RESULTS AND DISCUSSION

Three alleles (coded as A, B and C) were identified in exon 10 of CAPN3 gene in Barki sheep (as shown in Fig. 1), with frequency of 0.79, 0.14 and 0.07, respectively. These three alleles correspond to alleles 1, 2 and 3 which detected by Fang *et al.* (2013). Only three different genotypes AA, AB and AC were identified with frequency of 0.58, 0.29 and 0.13, respectively (Table 1).

The observed and expected frequencies of genotypes are shown in Table (1). In our study population, significant deviation from Hardy-Weinberg proportion was detected by χ^2 test (0.354). This means that the population was not in Hardy-Weinberg equilibrium for the genotypic polymorphism in exon 10 of CAPN3 gene. This might be due to the small number of genotyped lambs. Sire and the interaction between sire and CAPN3 genotype had no effect on all traits.

Association of the detected CAPN3 genotypes with growth and carcass traits was analyzed as shown in Table (2). High

significant association was observed between CAPN3 genotypes and post-weaning daily gain ($P < 0.005$). In addition, moderate significant association was found for CAPN3 genotypes with marketing weight and lean-meat% ($P < 0.05$). No associations were found between the rest of traits and CAPN3 genotype.

For the relative values of the studied traits (Table 2), animals with genotype AB had a higher mean of post-weaning daily gain (90.55±3.69 g/d) than animals with genotype AA (74.16±2.71 g/d) and BB (67.69±4.04 g/d). For marketing weight, AB animals had a higher mean (34.94±1.76) than animals with genotype AA (30.05±1.13 Kg) and AC (28.50±2.48 Kg). Animals with AB genotype also had higher lean-meat% value (50.84±1.39) compared to those animals with the AA genotype (45.63±2.71) and AC genotype (42.82±3.21). This means that the increased values for post-weaning daily gain, marketing weight and lean-meat% are associated with allele B, and the decreased values for these traits are associated with allele C.

In sheep breeding, post-weaning daily gain and marketing weight represent economically important traits. Lambs gaining rapidly are usually in a good condition, required reduced amount of feedstuffs, have a decreased risk of death and reach market weight at a younger age. Additionally, lamb lean meat% is a valuable retail product commanding a premium price in markets. Any selection tool identifies lambs that yield high marketing weight and lean meat% would be beneficial to the industry. Identification of causative genes that affect post-weaning daily gain and lean meat% will greatly enhance the progress towards that goal. Polymorphisms-in candidate genes and their associations with economic traits have been performed to ascertain the genetic basis of production traits and to develop marker assisted selection.

There are few studies linking CAPN3 genotypes to growth traits and market retail cuts of lambs. These studies reported that CAPN3 polymorphisms were associated with increased (11.8 to 13.3%) lamb birth weight (Chung *et al*., 2007), increased (1.8 to 2.4%) leg weight (Bickerstaffe *et al*., 2008) and increased shoulder weight and hot carcass weight (Fang *et al*., 2013). In our study, we found that polymorphisms in CAPN3 gene had significant association with post-weaning daily gain, marketing weight and lean meat%.

We assume that, like other agriculture animal species, this gene has potential role on muscle development, and animal which carrying allele B has a lower rate of muscle protein degradation however animal which carrying allele C has a higher rate of that. The callipyge sheep provided an evidence for a role of the calpain system in skeletal muscle growth. Muscle from the hindquarters of sheep having the callipyge gene is 30-40% larger than the corresponding muscle from normal sheep (Koohmaraie *et al*., 1995). Calpastatin activities in the hypertrophied muscles are

100-125% greater than calpastatin activities in the same muscles from normal sheep. This evidence suggests that decreased calpain activity, which mediated by increased calpaststin activity, is associated with decreased rate of muscle protein degradation and increased rate of muscle protein synthesis. In poultry, Maeda *et al.* (1991) found an association between muscle protein turnover rate and calpain activity in the muscle of Japanese quail.

CONCLUSION

It could be concluded that skeletal muscle CAPN3 has important regulation roles controlling muscle growth that reflect the combined activities of protein degradation**.** Thus, if any variation in coding regions of CAPN3 gene exists, it may account for phenotypic variations in muscle growth. Selection for allele B and against allele C of CAPN3 gene is expected to increase skeletal muscle mass which positively related to with high post-weaning daily gain, marketing weight and lean-meat% in carcasses.

SUMMARY

The calpain 3 (CAPN3) is a major intracellular protease encoded by CAPN3 gene and mainly expressed in skeletal muscle. This protease is necessary for normal muscle function, as mutations in CAPN3 result in increasing muscle protein degradation. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) was used to identify allelic and genotypic polymorphisms in exon 10 of CAPN3 gene for 24 Egyptian Barki lambs. Associations between the identified polymorphisms and growth and carcass traits were determined. Three alleles A, B and C were identified with frequency 0.79, 0.14 and 0.07, respectively. Only three genotypes were identified AA, AB and AC with frequency 0.58, 0.29 and 0.13, respectively. CAPN3 genotypes significantly affected post-weaning daily gain ($P < 0.005$), marketing weight ($P < 0.05$) and lean-meat% ($P<0.05$). Animals with AB genotype had higher means for post-weaning daily gain; marketing weight and lean-meat%. In contrast, animals with AC genotype had the lower means for these traits. Our findings suggest that the polymorphisms in CAPN3 may play a role affecting growth rate and lean meat% of carcass in Egyptian Barki sheep.

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Table (1): Allelic and genotypic frequencies of CAPN3 gene in Egyptian Barki sheep.

Parameters	Allelic frequencies			Genotypes and their frequencies		
Allele/Genotype (Number/Total)	A (38/48)	B (7/48)	C (3/48)	AA (14/24)	AB (7/24)	AC (3/24)
Observed frequency	0.79	0.14	0.07	0.58	0.29	0.13
Expected frequency	-	-	-	0.62	0.11	0.05

Table (2): Summary of means and significant and non-significant associations between CAPN3 genotypes and growth and carcass traits of Egyptian Barki lambs.

Traits	General Means	Means of Genotypes			P-values
		AA	AB	AC	
Birth weight (Kg)	3.58 ± 0.11	3.64 ± 0.14	3.36 ± 0.18	3.83 ±0.33	NS
Weaning weight (Kg)	17.22 ± 0.66	16.70 ± 0.81	18.64 ± 1.26	16.33±2.45	NS
Average daily gain (gm/d)	78.12 ± 2.57	74.16 ± 2.71^{ab}	90.55 ± 3.69^{a}	67.59 ± 4.04^{b}	**
Marketing weight (Kg)	31.29 ± 0.99	30.05 ± 1.13^{ab}	34.94 ± 1.76^{a}	28.50 ± 2.48^{b}	*
Empty body weight (Kg)	25.50 ± 0.74	24.84 ± 0.88	27.93 ± 1.29	22.95 ± 2.01	NS
Hot carcass weight (Kg)	14.04 ± 0.49	13.65 ± 0.60	15.34 ± 0.91	12.80 ± 1.47	NS
Cold carcass weight (Kg)	13.69 ± 0.47	13.40 ± 0.59	14.87 ± 0.94	12.33 ± 1.48	NS
Dressing %	44.82 ± 0.41	45.31 ± 0.44	43.86 ± 1.07	44.77 ± 0.77	NS
Neck %	7.08 ± 0.16	7.13 ± 0.21	6.92 ± 0.38	7.20 ± 0.31	NS
Shoulder %	19.71 ± 0.25	19.57 ± 0.21	19.84 ± 0.61	20.09 ± 1.30	NS
Rack %	24.60 ± 0.26	24.71 ± 0.35	24.144 ± 0.34	25.17 ± 0.88	NS
Loin %	6.65 ± 0.21	6.63 ± 0.24	6.91 ± 0.56	6.22 ± 0.53	NS
Flank %	4.02 ± 0.13	3.86 ± 0.17	4.15 ± 0.21	4.45 ± 0.55	NS
Leg %	34.23 ± 0.30	34.02 ± 0.38	34.90 ± 0.51	33.67 ± 0.90	NS
Tail %	3.28 ± 0.14	3.36 ± 0.20	3.15 ± 0.25	3.19 ± 0.48	NS
Lean-meat %	48.32 ± 1.31	45.63 ± 2.71^{ab}	50.84 ± 1.39^{a}	42.82 ± 3.21^{b}	*
Fat %	19.19 ± 1.10	18.05 ± 1.48	22.12 ± 2.07	17.71 ± 0.82	NS
Bone %	29.22 ± 0.49	29.15 ± 0.63	29.93 ± 1.00	27.95 ± 1.61	NS

Significance level * refers to significance at ($P < 0.05$) and ** refers to significance at ($P < 0.005$)

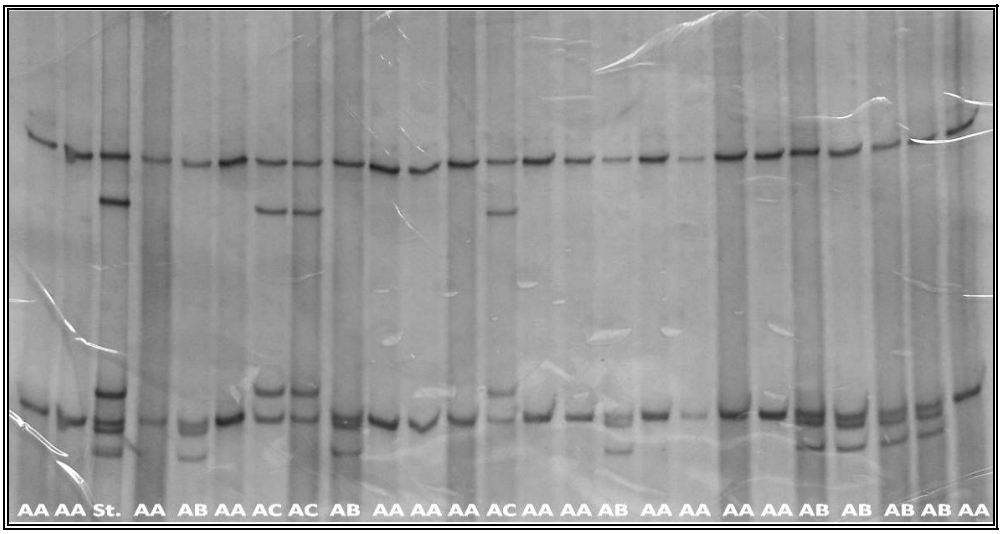

Fig. (1): PCR-SSCP analysis for exon 10 of CAPN3 gene in Egyptian Barki lambs.