Available online at www.easletters.com
(Special Issue 2S: Current Research in Biological Science)

EASL Entomology and Applied Science Letters

ISSN No: 2349-2864

Entomology and Applied Science Letters, 2016, 3, 2S:17-23

Polymorphism of CTSK Gene in Afshari×Booroola Merino Cross Lambs and its Association with Carcass Traits and Blood Factors

L. Mohammadi and T. Harkinezhad

Department of Animal Science, Faculty of Agriculture, Zanjan University, Zanjan, Iran
Corresponding E-mail: leila.mohammadi64@gmail.com

ABSTRACT

Cathepsin K (CTSK) which is a member of Lysosome cysteine protease gene family was selected as a candidate gene for fat deposition and it was shown that this lysosomal proteinase is an obesity marker. The aims of this study were to investigate the association of polymorphism in exon 6 and partial of intron 5 region in the Cathepsin K (CTSK) genes with carcass treats and blood factors in Afshari×Booroola Merino male lambs. In the present study, 97 Afshari× Booroola Merino male lambs of about the same age were used. Blood samples were taken from all animals for DNA extraction and blood components such as triglycerides, cholesterol, HDL, LDL and VLDL were measured. Primers for target area were designed; the length of target DNA was 500 bp. The target DNA was amplified using polymerase chain reaction (PCR) and products were conducted to single-strand conformation polymorphism (SSCP) and sequencing in order to identify polymorphism in this area of CTSK gene. Two different genotypes namely GA and GG were found according to the bands on non-denaturing polyacrylamide gel and their frequency were 26 and74 percent respectively. Comparing graphs obtained by sequencing each fragment was identifiedsingle nucleotide polymorphism (SNP). Results revealed the existence of a single nucleotide polymorphism at nucleotide 150 of target area. It was found that there is an association between this polymorphism and the tail weight, carcass and blood factors such as cholesterol and VLDL in other hand. The results show that the CTSK gene can be used in breeding programs to improve the quality of the carcass.

Key words: Afshari×Booroola Merino lambs, Carcass fat, Cathepsin genes, Polymorphism, SSCP

INTRODUCTION

Tenderness is considered to be the most important meat quality trait from consumers' point of view [1]. Intramuscular fat content and proteolysis during meat tenderization process are the main determinants of tenderness [2]. Cathepsin K (*CTSK*) is a member of the papain-like cysteine protease family. Members of this family are generally lysosomal enzymes although some, including *CTSK*are also found secreted from the cell. *CTSK*has potent proteolytic activities against several extracellular matrix components such as collagen I and II, elastase, osteonectin and osteopontin [3]. *CTSK*is most highly expressed in osteoclasts and its major recognized function is in the process of bone remodeling [4]. In the skeletal muscle, actin, myosin and associated proteins are the main targets of these peptidases and, therefore, they play a relevant role *post-mortem* in the process of conversion of muscles to meat [5]. Polymorphisms in cathepsins K (*CTSK*) have been related to pig performance traits such as average daily gain, feed/weight gain ratio, back fat thickness and weight of lean cuts [6]. In heart myocytes, cathepsin S degrades cathepsin K (*CTSK*) which is responsible for obesity-associated cardiac hypertrophy and ischemia-induced neovascularisation [7, 8]. *CTSK* has also recently been suggested as a new candidate gene for meat quality and fat content in pigs, and association between *CTSK* polymorphisms and back fat thickness has been reported [6]. Increased expression of the *CTSK* gene was observed in adipose tissue of experimentally obese mice and in human with obese phenotype [9, 10]. Moreover, *CTSK* deficient mice displayed significantly lower body weight and lower

level of fat deposition than wild type animals strengthening the role of this proteinase as a marker in human obesity [11]. The human *CTSK* genemaps on chromosome 1q21 (NCBI Human Genome Build 36.3; ttp://www.ncbi.nlm.nih.gov/projects/genome/guide/human/), a region that is syntenic with porcine chromosome4 (SSC4) [12], where several QTL for fat deposition and production traits have been identified [13].

According to these functions and data, *CTSK* can be considered a candidate gene for fatness and correlated traits in sheep as already shown for other cathepsin genes [14]. Here, we show that a novel single nucleotide polymorphism (SNP) in the porcine *CTSK* gene is associated with back fat thickness, blood components and other production traits in Afshari×Booroola Merino cross lambs.

MATERIALS AND METHODS

Breed description: sampling and DNA extraction

Blood samples (approximately 2-3 mL) were obtained from 97 unrelated Afshari×Booroola Merino cross lambs research-educational flock of Zanjan University were used and stored in ethylene diamine tetra acetic acid (EDTA)-coated tubes. Genomic DNA was extracted from 0.2 mL blood using a Phenol-chlophorm method according to manufacturer instructions. The quality and quantity of extracted DNA was measured on 0.8% agarose gel prepared in 0.5X TBE buffer (45 mMTris-base, 45 mM boric acid, 1 mM EDTA, pH 8.0), visualized with ethidium bromide (1.0 µg/mL) under ultraviolet light, and photographed.

PCR amplifications and detection of genetic variations using SSCP

The amplification of the exon 6 and partial of intron 5 region of the *CTSK* gene was achieved using two primers (5'-AGACCCTTGGTGGAGACTC-3') and (5'- ACGCTAGGAGACRCTCTG-3') targeting a fragment of 500bp which designed in NCBI website. The PCRs were done in a final volume of 16 pM of each primer, 100 ng of genomic DNA, 0.8 U Taq polymerase, 0.1 µM Taq PCR buffer, 200 µM of each dNTP, 2 mMMgCl,50 mMKCl. The thermal cycling profile consisted of an initial denaturation step of 95°C for 5 min, 35 cycles of denaturation at 94°C for 1min; annealing at 53°C for 50 seconds and extension at 72°C for 30 seconds followed by a final extension at 72°C for 5 min. Products of amplification were recognized by electrophoresis on 2% agarose gel stained with ethidium bromide. PCR products were mixed with 8 µL 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 µL. The mixture was denatured at 95°C for 5 min and then snap-chilled on ice [15]. The total volume was electrophoresed on 8% polyacrylamide gel, as described by [16]. The electrophoresis was performed in 0.5X TBE buffer at room temperature (18°C) and a constant 200 V for 3 h. Polyacrylamide gels were stained using silver nitrate according to the protocol described by [16]. PCR products from four different patterns of the *CTSK* gene were subjected to DNA sequencing.

For comparison of sequences with other sequences, we used the BLAST in the NCBI website and CLC Main Workbench 5 software.

Statistical analysis

Alleles and genotype's frequency and their accordance to Hardy-Weinberg equilibrium were calculated from POPGENE software (Yehet al., 1997). The observed and expected heterozygosity were calculated using Pop Gene software. The allelic and genotypic frequencies and observed and expected Nei'sheterozygosities (HE= 1 - Σ P2i, where Piis the frequency of allele i) were estimated using PopGene32 version 1.31 [17]. PopGene32 was also used to perform the Hardy-Weinberg equilibrium test. The associations of genotypes with body weight and blood factors were investigated using the *GLM* procedure of SAS 9.2 software. The following linear equations were applied to analysis the genetic affects the *CTSK* gene:

Equation for Blood factors such as: triglycerides, cholesterol, HDL, LDL and VLDL

$$y_{ij} = \mu + G_i + e_{ij}$$

Equation for biometric traits, live weight and body weight

$$y_{ijkl} = \mu + G_i + K_j + D_k + B(X_i - \mu) + e_{ijkl}$$

Where Y is the trait measured, μ is the population mean, G is the fixed effect the genotypes, D is the fixed effect of mother age, B is the covariate for birth weight, and e_{ijk} is the random error.

RESULTS AND DISCUSSION

PCR amplification of exon 6 and partial of intron 5 region of CTSK gene and SSCP Analysis

The exon 6 and partial of intron 5 region of *CTSK* gene (a fragment of 500bp in length) were successfully amplified in our first attempted by specific primers using NCBI website. All extracted DNAs from sheep blood samples yielded a specific single-band PCR product without any nonspecific bands (Figure 1).

Therefore, the PCR products were directly used for SSCP analysis. The allele variation in the *CTSK* gene was examined using a PCR-SSCP method. The non-denaturing gel electrophoresis allowed visualization of single-stranded DNA and SSCP band patterns. Two SSCP patterns were observed in the Afshari×Booroola Merino cross lambs (Figure 2).

The frequencies of the observed genotypes were 74 % and 26 % for GG and GA, respectively. Allele frequencies were 13 % and 87 % for A and G respectively (Table 1). Allele frequencies of the CTSK polymorphism in Hampshire were 12.5 % and 87.5 % for A and G respectively, and also allele frequencies of the CTSK for G allele more than A allele polymorphism in different pig breeds in Italian and Ukrainian [6, 18]. The frequencies of the observed genotypes were 0.90, 0.05 and 0.05, and 0.60, 0.35 and 0.05 for AA, AG and GG genotypes for Poltava Meat and Myrgorod in Ukrainian pig breeds.

The observed heterozygosity value was 0.26. The chi-square test was significant ($P \leq 0.01$) in deviation from Hardy-Weinberg equilibrium for the locus under study in the Afshari×Booroola Merino cross lambs population (Table 2).Observed heterozygosity and expected heterozygosity were 0.25 and 0.26 respectively. Balatsky et al. [18] was reported a relatively high level of polymorphisms in *CTSK* for Myrgorod breed (Obs Het = 0.35, Exp Het = 0.34).

Fontanesi et al. [6] investigated the polymorphism of *CTSK* gene in seven pig breed. There are two allele in the intron 4 of *CTSK* gene with the frequencies of 0.944 (allele g.15G) and 0.056 (allele d.15A), 0.897 (allele g.15G) and 0.103 (allele d.15A), 0.925 (allele g.15G) and 0.075 (allele d.15A), 1.000 (allele g.15G) and 0.000 (allele d.15A), and 0.708 (allele g.15G) and 0.292(allele d.15A) in Italian Large White, Italian Durco, Italian Landrace, Pietrain, Belgian Landrace and Meishan breeds respectively. In this study two allele observed similarity results of Fontanesi et al. [6].

Sequence results

Sequence analysis using the Chromas lite and CLC Main Workbench 5 software revealed SNPs among all examined *CTSK* sequences (Figure 3). The results of the sequence analysis confirmed the SSCP results. The results of this study partially agree with the result of Funtanesi et al. [6] who they reported single nucleotide polymorphism in the intron 4 of *CTSK* gene.

Association of exon 6 and partial of intron 5 region of CTSK gene polymorphism with birth weight and live weight

According to the association study result, there is not any significant association between genotypes with live weight and birth weight in "Afshari×Booroola Merino cross lambs population" (Table 3). Other relationships between the *CTSK* gene and back fat thickness lose weight, mean of daily weight and Feed Conversion Ratio (FCR) in Italian Duroc pigs reported by Fontanesi et al. [6]. They result show that there is significant difference between SNPs in the intron 4 of *CTSK* gene and the traits ($P<0.05$). Xiao et al. [10] reported that gain weight affected by the *CTSK* gene. Russo et al., (2008) have identified significant difference between single nucleotide polymorphism in *CTSB* and *CTSD* gene with back fat thickness, lose weight, mean of daily weight and Feed Conversion Ratio (FCR) in Italian Large White Pigs.

Association of exon 6 and partial of intron 5 region of CTSK gene polymorphism with biometric traits

The results of GLM show that no association between the biometric traits and genotype in exon 6 and partial of intron 5 region of *CTSK* gene (Table 4). According to the results, in all biometric traits GG genotype had a higher

value that GA genotype. In the previouse study, there are not any resarch about association between biometric traits and *CTSK* gene.

Association of exon 6 and partial of intron 5 region of CTSK gene polymorphism with blood factors

General linear models revealed that the GA genotype (3.84 mmol/l) was compared GG genotype (3.79 mmol/l) was associated with VLDL. The effect of the GG and GA genotypes on other blood factors such as cholesterol, HDL, LDL was non-significant (Table 5). There is not any research about the *CTSK* gene with blood parameters such as HDL, VLDL in farm animal.

Association of exon 6 and partial of intron 5 region of CTSK gene polymorphism with carcass character

Association analysis of carcass characters revealed that only two characters (tail and total fat) had significant difference between two genotype and there are not among other carcass character (Table 6).

According to the results of analysis, the percent average of tail for GA and GG genotypes were 4.8 and 6.6 respectively and the average of total fat for GA and GG genotype were 10.01 and 12.06 respectively. Among all carcass traits only two traits (percentage of tail and percentage of total fat) have shown significant difference and there are not any significant difference between other carcass traits. The results shown that the average of GG genotype had higher value in all traits of carcass. The results of Fontanesi et al. [6] showed that polymorphisms in the *CTSZ* gene had a significant difference with the weight of leg meat trait ($p<0.01$). Also they results show that in the Italian white pig there is a positive correlation between growth rate and back fat thickness. *CTSK* gene identified as one of the new markers for fat in adipose tissue in human, mouse and pig [9]. The effect of *CTSK* gene on weight gain was significant [10].

Fontanesi et al. [19] identified single nucleotide polymorphisms (SNPs) associated with backfat thickness in Italian White pigs using genome wide association study (GWAS). According their results, there are four single nucleotide polymorphisms with the most significant effect and located in chromosomes 6, 7 and 9.

Results of sequence comparison

The results of percent similarity between the sequence of *CTSK* gene in "Afshari×Booroola Merino cross lambs population" with the sequence of sheep, Cow, Pig, Human and Mouse with access number in NCBI database are shown in the table 7. The similarity with is 99.79%, 78.7%, 71%, 80% and 80% with sheep, Cow, Pig, Human and Mouse respectively.

Table 1. Observed allele and genotypic frequencies for CTSK locus.

Allele Frequency (%)		Genotype frequency (%)	
A	G	GA	GG
13	87	26	74

Table 2. Summary of heterozygosis statistics and genetic variation statistics for CTSK locus.

Locus	ObsHet	ExpHet	Nei	Na	Ne	I
INHa	0.26	0.25	0.24	2	0.24	0.66

Na = observed number of alleles; Ne = Effective number of alleles; I = Shannon's Information index.

Table 3. Effect of the CTSK gene genotype on birth and live weight in "Afshari×Booroola Merino cross lambs population".

Trait	Genotype		P-Value
	GA	GG	
Birth Weight (kg)	5.61±0.17	5.32±0.11	ns
Live Weight(kg)	54.75±0.16	57.1±1.26	ns

Table 4. Effect of the CTSK gene genotype on biometric traits in "Afshari×Booroola Merino cross lambs population".

Trait	Genotype		P-Value
	GA	GG	
Height at Withers (cm)	69.52±0.70	72±0.40	ns
Chest Girth (cm)	97.73±1.62	102.51±0.91	ns
Back Length (cm)	49.18±0.87	50±0.52	ns
Legs Distance (cm)	18.57±0.60	19±0.36	ns

Table 5. Effect of the CTSK gene genotype on blood factors in "Afshari×Booroola Merino cross lambs population".

Trait	Genotype		P-Value
	GA	GG	
Cholesterol (mmol/l)	49.08±2.6	47±1.62	ns
HDL (mmol/l)	27.19±0.64	24.25±0.35	ns
LDL (mmol/l)	20.26±0.33	19.5±1.8	ns
VLDL (mmol/l)	3.84±0.22[a]	3.79±0.13[b]	0.05

Table 6. Effect of the CTSK gene genotype on carcass characters in "Afshari×Booroola Merino cross lambs population".

Trait	Genotype		P-Value
	GA	GG	
Carcass Weight (%)	43.37±0.35	42.76±0.27	ns
Carcass Fat Thickness (cm)	1.5±0.2	1.8±0.1	ns
Diameter of Carcass Muscle(cm)	2.3±0.1	2.32±0.1	ns
Leg (%)	31.4±0.64	31±0.51	ns
Shoulder (%)	17.22±0.53	17±0.3	ns
Neck (%)	8.3±2.3	10±1.2	ns
Waste (%)	4.6±0.3	3.6±0.23	ns
Tail (%)	4.8±0.4[a]	6.6±0.4[b]	0.05
Total Fat (%)	10.01±0.22[a]	12.6±0.71[b]	0.05

Table 7. Percent similarity of exon 6 and partial of intron 5 region of CTSK gene in "Afshari×Booroola Merino cross lambs population" with other type of spices.

Access Number in NCBI	Type of Spices	Percent Similarity
ENSOARG00000020869	Sheep (ovisaries)	99.7
NM 001075725.1	Cow	78.7
CU855548.6	Pig	71
AL691515.9	Human	80
AL731703.16	Mouse	80

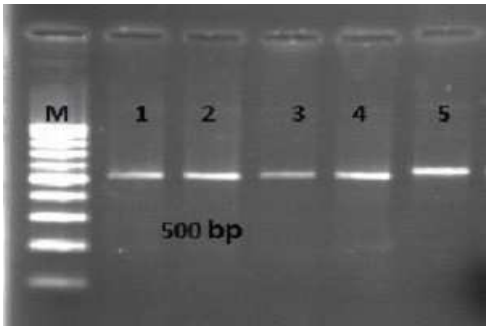

Figure 1. PCR bands of CTSK gene

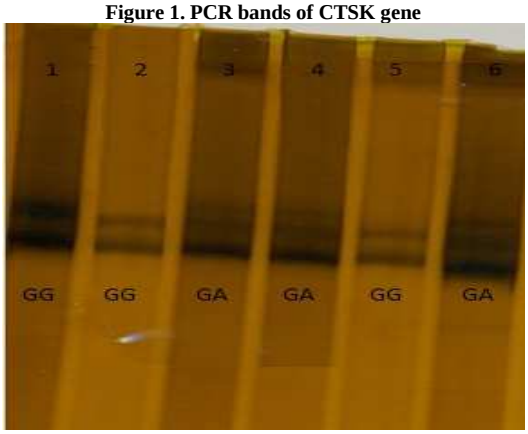

Figure 2. SSCP polymorphism of Afshari×Booroola Merino of CTSK gene, two different PCR-SSCP patterns (genotype) were identified

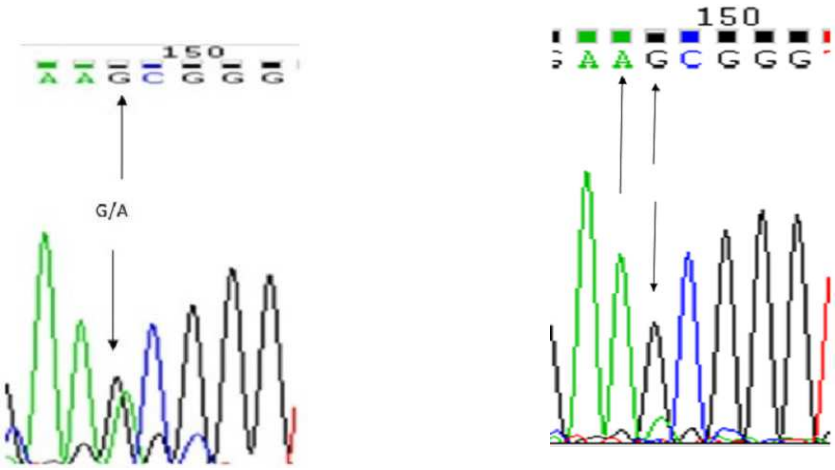

Figure 3: SNPs profile of CTSK gene in "Afshari×Booroola Merino cross lambs population"

CONCLUSION

This is the first study which investigates *CTSK* gene polymorphisms in "Afshari×Booroola Merino" cross lambs. Very little information is currently available with which to compare various Iranian or other sheep breeds. Breeding programs in most research centers in Iran have been based solely on phenotypic characters. The current study confirmed the importance of molecular studies in addition to morphological data for the detection of genetic variation among individuals when selecting diverse parents with which to construct a new population. Additional research is needed to characterize the completely ovine *CTSK* gene variation across an extended region of the gene and in a large variety of sheep breeds from around the world. Result of this study confirmed that this gene is as a new maker in adipose tissues in farm animals. Further studies with numerous markers and genes in the *CTSK* region and other breeds will be required in order to understand *CTSK* genetics in sheep and clarify the genetic background.

Acknowledgments
The authors would like to acknowledge the Dr. Ali Olfati (Chair man of this special issue, from University of Tabriz, A.olfati65@gmail.com; +98919 596 6273), thank you for keeping me on track, helping me to resolve issues and assisting me to get my dissertation completed.

REFERENCES

[1] Miller MF, Carr MA, Ramsey CB, Crockett KL, Hoover LC. *J. Anim. Sci*. **2001**, 79 (12): 3062-3068.
[2] Sentandreu MA, Coulis G, Ouali A. *Trends. Food. Sci. Tech*. **2002**, 13: 400-421.
[3] Lecaille F, Choe Y, Brandt W, Li Z, Craik CS, Bromme D. *Biochemistry*. **2002**, 41: 8447–8454.
[4] Buhling F, Reisenauer A, Gerber A, Kruger S, Weber E, Bromme D, Roessner A, Ansorge S, et al. *J. Pathol*. **2001**, 195: 375–382.
[5] Virgili R, Schivazappa C, Parolari G, Soresi Bordini C, Degni M. *J. Food. Biochem*. **1998**, 22:53-63.
[6] Fontanesi L, Scotti E, Buttazzoni L, Dallolio S, Davoli R, Russo V. *Mol. Biol. Report*. **2010**, 3: 491-495.
[7] Hua Y, Zhang Y, Dolence J, Shi GP, Ren J, Nair S. *Diabetes*. **2013**, 62 (2): 498-509.
[8] Jiang H, Cheng XW, Shi GP, Hu L, Inoue A, Yamamura Y, Wu H, Takeshita K, X, et al. *Nat. Commun*. **2014**, 5: 38-42.
[9] Chiellini C, Costa M, Novelli S, Amri E, Benzi L, Bertacca A, Cohen P, DEL Prato S, FriedmanJM, Maffei M. *J. Cell. Physiol*. **2003**, 195: 309–321.
[10] Xiao Y, Junfeng H, Tianhong L, Lu W, Shulin C, Yu Z, Xiaohua L, Weixia J, Sheng Z, Yanyun G, Guo L, Min L. *J. Clin. Endocrinol. Metab*. **2006**, 91:4520–4527.
[11] Funicello M, Novelli M, Ragni M, Vottari T, Cocuzza C, Soriano-Lopez J, C. Chiellini C, Boschi F, Marzola P, et al.. *PLoS. One*. **2007**, 2: 683.

[12] Moller M, Berg F, Riquet J, Pomp D, Archibald A, AndersonS , et al. *Mamm. Genome*. **2004**, 15:717–731.
[13] Cepica S, Stratil A, Kopecny M, Blazkova LP, Schro JR, Davoli R, Fontanesi L, Reiner G, Bartenschlager H, Moser G, Geldermann H. *J. Anim. Breed. Genet*. **2003**, 120: 28–37.
[14] Russo V, Fontanesi L, Scotti E, Beretti F, Davoli R, et al. *J. Anim. Sci*. **2008**, 86: 3300-3314.
[15] Pipalia DL, Joshi CG, Rank DN, Brahmkshtri BP. *Indian. J. Anim. Sci*. **2004**, 74: 637-639.
[16] Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR. *J. Clin. Microbiol*. **1982**, 16: 473-477.
[17] Yeh FC, Boyle T, Yang R. University of Alberta, Edmonton. **1997**.
[18] Balatsky VN, Pochernyaev KF, Buslyk TV, DYKANOS, Korinnyi SN, Pena RN, DORAN O. Glob. *J. Anim. Breed. Genet.* **2015**, 3 (3): 117-124.
[19] Fontanesi L, Schiavo G,Galimberti G, Calo DG, Scotti E, Martelli PL, Buttazzoni L, Casadio R, Russo V. BMC. *Genomics*. **2012**, 15: 13:583.