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Association between CAST and MSTN gene polymorphisms with growth traits in Awassi sheep

Mervan Bayraktar[1, *], Omer Shoshin[2]
[1]*Dept. of Animal Science, Cukurova University, Adana, Turkey*
[2]*Dept. of Veterinary collage, Physiology Science, Kirkuk University, Kirkuk, Iraq*
[*]*Corresponding author: mbayraktar@cu.edu.tr*

Abstract

This study aims to identify the association between CAST/*MspI* and MSTN/*HaeIII* polymorphism with growth traits in Awassi sheep. A total of 129 (male and female) Awassi sheep were used in the study. Growth traits (Body weight BW, body length BL, chest depth CD, heart girth HG, and withers height WH) were taken from animals at one year old. PCR-RFLP analysis was used to detect CAST polymorphism (Exon 1C/1D) and MSTN (Exon 3) genes. Three genotypes (MM, MN and NN) were observed for CAST/*MspI* polymorphism with allele and genotype frequency 0.78(M) and 0.22(N); 0.70(MM), 0.16(MN), and 0.14(NN). Two genotypes (MM and mm) were found for MSTN/*HaeIII* polymorphism with allele and genotype frequency as 0.10(M) and 0.90(N); 0.10(MM) and 0.90(mm). The genes were in agreement with Hardy-Weinberg equilibrium ($p>0.05$). The association analysis showed an association between CAST/*MspI* polymorphism and BW, BL, CD, and HG ($P<0.05$). The MM genotypes had the highest BW compared to MN and NN genotypes. At the same time, the MN genotypes had the highest BL, CD, and HG compared to MM and NN genotypes. No association was found between MSTN/*HaeIII* polymorphism and growth traits.

Keywords: Awassi sheep; CAST; growth traits; MSTN; polymorphism.

1. Introduction

Sheep are considered the most suitable agricultural animals for grazing in dry and harsh areas. These areas may not be ideal for other farm animals due to sheep's ability to graze and adapt to environmental conditions. Iraqi sheep belong to the fat-tailed Asian sheep and include Karadi, Arabi, and Awassi. Awassi is a triple-purpose sheep (dairy, meat, and wool) and constitutes about 60% of native sheep breeds in Iraq (Al Qasimi et al., 2019; Al-Barzinji & Ameen, 2019). Sheep breeding in Iraq is still taking the traditional methods that depend on grazing in poor areas, so productivity is low. Therefore, it is necessary to follow new management methods nutrition, improve environmental conditions and follow new genetic improvement methods. Increasing the productivity of Awassi requires genetic improvement methods, identifying genetic variation, and determining genes that affect growth and production traits (Al-Salihi et al., 2017; Eghbalsaied et al., 2016; Al-Salameen et al., 2014; Ghani et al., 2021).

Calpastatin (CAST) and myostatin (MSTN) genes directly affect the growth traits in sheep. CAST gene is an endogenous protein that inhibits the effect of calpain (Balcıoğlu *et al.*, 2014). Ovine CAST gene is located at chromosome 5 contains 32 exons, 2.701 bp transcript length, and 786 residues of translation length (No: ENSOART00000019281.1). CAST gene plays an essential role in muscle development and meat tenderness after slaughter (Gabor *et al.*, 2009). MSTN, also known as growth and differentiation factor 8 (GDF8), plays to inhibit muscle growth by preventing muscle fibers formation (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; McPherron & Lee, 1997). Ovine MSTN gene is located on chromosome 2 (BTA2) and consists of three exons and two introns (Bellinge *et al.*, 2005; Jeanplong *et al.*, 2001; O'Rourke, 2010). The MSTN gene mutations are associated with the double-muscled phenomenon in various mammalian species (Casas *et al.*, 1998; Clop *et al.*, 2006; Mosher *et al.*, 2007; Schuelke *et al.*, 2004). The MSTN gene is related to growth traits in sheep.

The current study aimed to determine the association between CAST and MSTN polymorphisms with growth traits in Awassi sheep.

2. Material and methods

2.1 Experimental animals

A total of 129 sheep (ram n=47, ewe n=82) of the Awassi breed were used in this study. The animals were raised on a private farm. The farm is located south of Kirkuk city and 50 km away from the city center. Growth trait data of BW, BL, CD, HG, and WH were taken from sheep at one year old.

2.2 Sample collection and DNA extraction

Genetic analysis was carried out in the molecular genetics laboratory at the College of Veterinary, University of Kirkuk. The blood was collected from the jugular vein using ethylenediamine tetra-acetic acid (EDTA) tubes and stored at -20°C. Genomic DNA was extracted from whole blood by using the phenol-chloroform methods. The primer sequence of the CAST and MSTN gene was given in (Table 1). The PCR was done in a reaction volume of 10 µL, contains 2 µL (50ng) DNA, 2.5 µL of PCR Master Mix (GoTaq® G2 Green Master Mix, Promega, USA), 0.25 µL for each primer (10 µ mol) and 5 µL distilled water. PCR conditions for the CAST and MSTN genes are given in (Table 2).

2.3 PCR-RFLP method

CAST locus was digested with *MspI* enzyme and MSTN digested by *HaeIII* enzyme (Promega, USA). The mix consisted of 5 µL PCR product, 3.5 µL distilled water, 1 µL 10X buffer, and 0.5 µL restriction enzyme (Total of 10 µL). Digestion products were separated on 2% agarose gel at 95 V for 60 min. The gel was stained with ethidium bromide and used a 100bp DNA marker (Promega, USA). The results were checked under ultraviolet light.

Table 1. The primer sequences of CAST and MSTN gene

Gene	primer sequences	position	Source
CAST	F/5’TGGGGCCCAATGACGCCATCGATG-3’ R/5’GGTGGAGCAGCACTTCTGATCACC-3’	Exon 1C/1D	Palmer *et al.* (1998)
MSTN	F/5’CCGGAGAGACTTTGGGCTTGA-3’ F/5’TCATGAGCACCCACAGCGGTC-3’	Exon 3	Smith *et al.* (1997)

2.4 Statistical Analysis

The allele and genotype frequency of the genes and the Chi-square test χ^2 were calculated by popgen32 (ver.1.32). Growth traits were analyzed using the General Linear Model (GLM) of

Minitab 16. The least-squares means were compared using Tukey, the least significant difference test.

The general linear model was:

Yijk = μ + αi + βj + αβij + eijk

Yijk: traits measured; μ: overall mean for each trait; αi: sex effect; βj: genotypes effect; αβij: interaction between genotype and sex; eijk: random error

3. Results

3.1 CAST/*MspI* polymorphism

622 bp of PCR product was amplified. Three genotypes (MM, MN, and NN) were obtained (Figure 1). MM genotype was 336 bp and 286 bp; MN genotype was 622 bp, 336 bp, and 286 bp; NN genotype was 622 bp. Chi-square χ^2 test showed agreement to Hardy-Weinberg equilibrium (p>0.05) (Tablo 3). The allele and genotype frequency was 0.78(M) and 0.22(N); 0.70(MM), 0.16(MN), and 0.14(NN). M Allele showed a high frequency from the N allele.

3.2 MSTN/*HaeIII* polymorphism

337 bp of PCR product was amplified. Two genotypes (MM, and mm) were observed. MM genotype was 337 bp, whereas mm genotype was 131 and 123 (Figure 2) bp. χ^2 test showed agreement to Hardy-Weinberg equilibrium (p>0.05) (Table 3). The allele and genotype frequency was 0.10(M) and 0.90(m); 0.10(MM) and 0.90(mm) respectively.

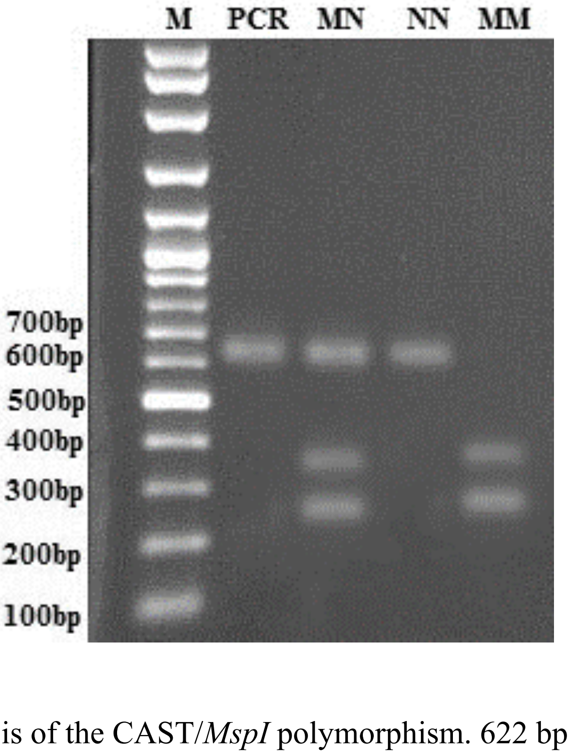

Fig. 1. PCR-RFLP analysis of the CAST/*MspI* polymorphism. 622 bp PCR fragment; 622 bp, 336 bp, and 286 bp for MN genotype; 622 bp for NN genotype; 336 bp and 286 bp for MM genotype

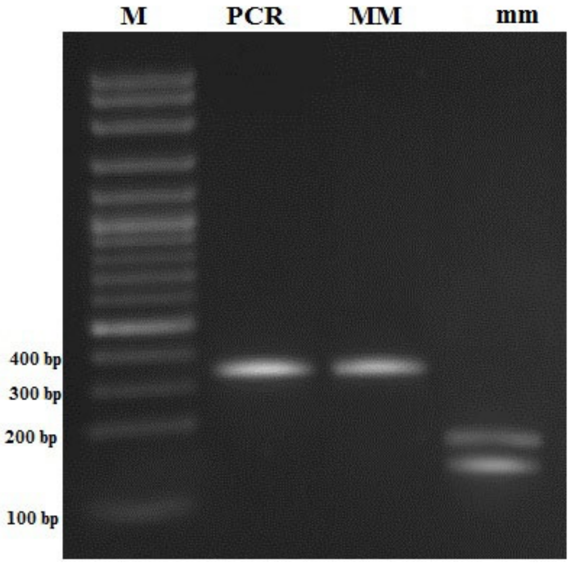

Fig. 2. PCR-RFLP analysis of the MSTN/*HaeIII* polymorphism. 337 bp PCR fragment; 337 bp for MM genotype; 131 bp and 123 bp for mm genotype

Table 2. Allele and genotype frequencies of CAST/*MspI* and MSTN/*HaeIII* polymorphisms

Gene	Allele Frequency		Genotype Frequency			χ^2
	M	N	MM	MN	NN	39.76
CAST	0.78	0.22	0.70	0.16	0.14	
	M	m	MM	Mm	mm	
MSTN	0.10	0.90	0.10	0.00	0.90	129

3.3 Association analysis

3.3.1 Association between polymorphisms and growth traits

Association analysis showed a significant effect of CAST locus on the growth traits ($P<0.05$). The animals with MM genotypes had the highest BW from MN and NN genotypes (Table 3). The MN genotypes had the highest BL, CD, HG, and WH than MM and NN genotypes. A significant interaction was observed between body length, chest depth, and Heart girth with sex. MSTN locus had no significant effect on growth traits ($P>0.05$) (Table 4).

Table 3. Association analysis between CAST genotypes and growth traits

Traits	Genotypes (mean ± standard error)			p-value	G*S
	MM	MN	NN		
Body weight, kg	43.22± 0,097^{a}	42,68±0,131^{b}	42.86±0.135^{ab}	0.003*	0.383
Body length, cm	55.10±0.119^{b}	55.67±0.159^{a}	55.01±0.165^{b}	0.006*	0.016
Chest depth, cm	30.00±0.105^{ab}	30.38±0.140^{a}	29.87±0.145^{b}	0.028*	0.064
Heart girth, cm	87.96±0.095^{ab}	88.29±0.128^{a}	87.84±0.132^{b}	0.033*	0.002
Wither height, cm	67.51±0.098	67.87±0.132	67.80±0.138	0.063	0.764

G*S: interaction between genotype and sex; * $P<0.05$

Table 4. Association analysis between MSTN genotypes and growth traits

Traits	Genotypes (mean ± standard error)		p-value	G*S
	MM	mm		
Body weight. kg	42.92±0.090	42.92±0.108	0.970	0.562
Body length. cm	55.22±0.110	55.30±0.132	0.650	0.389
Chest depth. cm	30.15±0.097	30.01±0.116	0.353	0.228
Heart girth. cm	88.02±0.088	88.04±0.106	0.866	0.642
Wither height. cm	67.73±0.092	67.72±0.111	0.947	0.343

G*S: interaction between genotype and sex

4. Discussion

CAST gene directly affects growth traits and meat characteristics. In this study, we identified three genotypes (MM. MN and NN) of CAST/*MspI* polymorphism, with allele and genotype frequency 0.78(M) and 0.22(N); 0.70(MM), 0.16(MN), and 0.14(NN). Previous studies reported that the M allele is more frequent than the N allele in most sheep breeds (Table 5). We also found a high frequency for the M allele. Nassiry *et al.* (2006) determined the A allele frequency in the Kurdi sheep as 0.78. Khederzadeh *et al.* (2016) showed the A allele frequency as 0.78 in Zandi sheep. Also, Jawasreh *et al.* (2019a) noted M allele frequency in Awassi sheep as 0.77. Eftekhari *et al.* (2006) observed M allele frequency in Karakul sheep as 0.79. The association analysis showed a significant association between CAST polymorphism and growth traits. The MM genotypes had the highest BW compared to MN and NN genotypes. In contrast, the MN genotypes had the highest BL, CD, and HG from MM and NN genotypes. Nassiry *et al.* (2006) observed the association between CAST polymorphism and average daily gain from birth to weaning in Kurdi sheep. Byun *et al.* (2008) reported the CAST genes effect on birth weight in New Zealand Romney sheep. Sutikno *et al.* (2011) found no association between CAST polymorphism and bodyweight of Indonesian native sheep. Chung & Davis (2012) found an association between CAST polymorphism and growth traits in Playpay, Targhee, and crossbreed sheep. Nikmard *et al.* (2012) did not find an association between CAST polymorphism and growth traits in Afshari sheep. Khan *et al.* (2012) showed an association between CAST polymorphism and average daily gain in Balkhi and Kajli sheep. Yilmaz *et al.* (2014) determined the effect of CAST on the average daily gain in Kıvırcık lambs. Ibrahim *et al.* (2015) did not observe the effect of CAST on the growth traits in Barki sheep. Gorlov *et al.* (2016) detected an association between CAST polymorphısm and growth traits in Salsk sheep. Ihsan *et al.* (2016) revealed an association between CAST polymorphısm and average daily gain in Indonesian thin tail sheep. Jawasreh *et al.* (2017) confirmed a significant association between CAST polymorphism and final body weight and average daily gain in Awassi sheep. Bayram *et al.* (2019) did not find a CAST effect on body weight in Akkaraman lambs. Afanasyeva *et al.* (2019) determined the association between CAST polymorphism and average daily weight gain in the West Siberian mutton breed. Jawasreh & Ismail (2019b) demonstrated the CAST effect on final body weight in Awassi sheep. Machado *et al.* (2020) identified the association of CAST with growth traits in Ines sheep. Al-Barzinji & Ameen (2019) showed that lambs with AB genotype had a higher body weight at all ages and higher average daily gain.

MSTN gene inhibits muscle cell growth. When loss of the vital function of the MSTN gene causes the double-muscled in the sheep (Broad *et al.*, 2000).

We detected two genotypes (MM and mm) of MSTN locus, with allele and genotype frequency 0.10(M) and 0.90(N); 0.10(MM) and 0.90(mm). Soufy *et al.* (2009) observed three genotypes in Sanjabi Sheep with genotype frequency 0.02(MM), 0.01(Mm), and 0.97 (mm). Sahu *et al.* (2017) reported two genotypes (MM and Mm) in Madras Red and Mecheri sheep breeds at G5622C locus in exon 3 of MSTN/*MspI* polymorphism with genotype frequency as 0.41(MM) and 0.58(Mm); 0.48(MM) and 0.51(Mm) respectively. In contrast, two genotypes (Mm and mm) were shown in Kordi, Kalehkoohi, Farahani, Mehraban, and Teleorman sheep breeds (Akbari *et al.*, 2015; Ebrahimi *et al.*, 2014; Jamshidi *et al.*, 2014; Lazar *et al.*, 2016; Shariatzadeh *et al.*, 2014). Most studies confirm that the MSTN/*HaeIII* at exon 3 is monomorphic in the different sheep breeds (Table 6). The reason may be the small sample size, environmental effect, geographical position, and mating strategies. Association analysis showed no significant association between MSTN/*HaeIII* polymorphism and growth traits (P>0.05). There are not many association studies because of the monomorphic at the MSTN/*HaeIII* gene locus. Sahu *et al.* (2017) found an association between genotypes at G5622C locus in exon 3 of MSTN/*MspI* site and body weight at 9 and 12 months.

Table 5. Distribution of Allele Frequency in the CAST gene at some sheep breeds

References	Breed	N	Allele frequency	
Nassiry *et al.* (2006)	Kurdi sheep	84	0.78(A)	0.16(B) 0.06(C)
Eftekhari *et al.* (2006)	Karakul sheep	100	0.79(M)	0.21(N)
Mohammadi *et al.* (2008)	Arabic sheep	111	0.85(A)	0.15(B)
Gabor *et al.* (2009)	Tsigai sheep	58	0.91(M)	0.09(N)
	Improved valachian	19	0.97(M)	0.03(N)
Szkudlarek-Kowalczyk *et al.* (2011)	Polish Merino	82	0.76(M)	0.24(N)
	Berrichon du Cher	41	0.93(M)	0.7(N)
Sutikno *et al.* (2011)	Local Sheep	264	0.86(M)	0.14(N)
Nanekarani *et al.* (2011)	Atabi sheep	120	0.81(A)	0.19(B)
Dehnavi *et al.* (2012a)	Zel sheep	200	0.85(M)	0.15(N)
Chung & Davis (2012)	Polypay	116	0.53(A)	0.47(B)
	Targhee	110	0.18(A)	0.82(B)
Gharahveysi *et al.* (2012)	Zel sheep	100	0.75(M)	0.25(N)
Khan *et al.* (2012)	Balkhi	300	0.88(M)	0.12(N)
	Kajli	300	0.86(M)	0.14(N)
Azari *et al.* (2012)	Dalagh sheep	110	0.55(A)	0.45(B)
Ranjbari *et al.* (2012)	Makoei Sheep	100	0.63(A)	0.36(B) 0.01(C)
Suleman *et al.* (2012)	Thalli	100	0.90(M)	0.10(N)
	Lohi	100	0.87(M)	0.13(N)
Ata (2012)	Çine çaparı	97	0.74(M)	0.26(N)
	Karya sheep	90	0.54(M)	0.46(N)
Nikmard *et al.* (2012)	Afshari sheep	51	0.74(M)	0.26(N)
Balcıoğlu *et al.* (2014)	Kangal	31	0.92(M)	0.08(N)
	Awassi	26	0.59(M)	0.41(N)

Nanekarani & Goodarzi (2014)	Lori sheep	120	0.63(A)	0.37(B)
Avanus (2015)	Kıvırcık	25	0.70(M)	0.30(N)
	Karakul	15	0.73(M)	0.27(N)
Ibrahim *et al.* (2015)	Barki sheep	42	0.62(M)	0.13(N) 0.10(O) 0.15(P)
Georgieva *et al.* (2015)	Shumen	121	0.92(M)	0.08(N)
Mahrous *et al.* (2015)	Barki	20	0.68(M)	0.32(N)
	Rahmani	20	0.80(M)	0.20(N)
Gorlov *et al.* (2016)	Soviet merino	72	0.88(M)	0.12(N)
	Salsk	108	0.89(M)	0.11(N)
Bozhilova-Sakova & Dimitrova (2016)	Karakachan sheep	25	1.00(M)	-
Khederzadeh *et al.* (2016)	Zandi sheep	100	0.78(A)	0.22(B)
Jawasreh *et al.* (2017)	Awassi	80	0.49(M)	0.51(N)
Kaplan & Atalay (2017)	Kıvırcık	100	0.90(M)	0.10(N)
Ibrahim & Kali (2017)	Awassi	40	0.86(M)	0.14(N)
Kulikova *et al.* (2018)	Tuvan steppe type	51	0.89(M)	0.11(N)
	Tuvan mountain type	100	0.88(M)	0.12(N)
Bayram *et al.* (2019)	Akkaraman	374	0.90(M)	0.10(N)
Gaitonde (2018)	Deccani	50	0.75(M)	0.25(N)
	Madgyal	50	0.62(M)	0.38(N)
Pomitun *et al.* (2019)	Kharkiv	47	0.83(M)	0.17(N)
Afanasyeva *et al.* (2019)	West Siberian mutton breed	100	0.84(M)	0.16(N)
Montes *et al.* (2019)	Valle del Cauca	150	0.91(M)	0.09(N)
	Sucre	150	0.92(M)	0.08(N)
Jawasreh *et al.* (2019a)	Awassi	87	0.77(M)	0.23(N)
Jawasreh & Ismail (2019b)	Awassi	31	0.49(M)	0.51(N)
Al-Barzinji & Ameen (2019)	Awassi	52	0.12(A)	0.88(B)

Table 6. Distribution of Allele Frequency in the MSTN gene at some sheep breeds

References	**Breed**	**N**	**Allele frequency**	
Soufy *et al.* (2009)	Sanjabi	150	0.03(M)	0.97(m)
Dehnavi *et al.* (2012b)	Zel	200	-	1.00(m)
Azari *et al.* (2012)	Dalagh	110	-	1.00(m)
Zare & Mirhosseini (2013)	Karakul	100	-	1.00(m)
Elkorshy *et al.* (2013)	Barki	25	-	1.00(m)
	Rahmani	24	-	1.00(m)
	Saidi	25	-	1.00(m)
	Najdi	21	-	1.00(m)
	Harri	22	-	1.00(m)
	Ossimi	23	-	1.00(m)
Nada *et al.* (2013)	Barki	25	-	1.00(m)
	Ossimi	48	-	1.00(m)

Ebrahimi *et al.* (2014)	Kalehkoohi	96	0.20(M)	0.80(m)
Shariatzadeh *et al.* (2014)	Farahani Sheep	86	0.11(M)	0.89(m)
Jamshidi *et al.* (2014)	Mehraban	120	0.03(M)	0.97(m)
Georgieva *et al.* (2015)	Shumen	121	-	1.00(m)
Akbari *et al.* (2015)	Kordi sheep	58	0.08(M)	0.92(m)
Bozhilova-Sakova *et al.* (2016)	Karakachan	25	-	1.00(m)
Dimitrova *et al.* (2016)	Bulgarian Merino	32	-	1.00(m)
Othman *et al.* (2016)	Egyptian sheep	171	-	1.00(m)
Khederzadeh *et al.* (2016)	Zandi sheep	100	-	1.00(m)
Lazar *et al.* (2016)	Teleorman	105	0.42(M)	0.58(m)
Sahu *et al.* (2017)	Madras Red	127	0.71(M)	0.29(m)
	Mecheri	105	0.74(M)	0.26(m)
Bozhilova-Sakova & Dimitrova (2017)	France	30	-	1.00(m)
Khederzadeh *et al.* (2017)	Shirazi Sheep	102	-	1.00(m)
Dimitrova *et al.* (2017)	Karnobat Merino	35	-	1.00(m)
Dimitrova *et al.* (2019)	Ascanian merino	31	-	1.00(m)
	Caucasian merino	30	-	1.00(m)
Al-Barzinji & Ameen (2019)	Awassi	52	-	1.00(B)

5. Conclusion

This study found a significant association between the CAST/*MspI* and MSTN/*HaeIII* polymorphisms and growth traits in Awassi sheep. As the genes showed polymorphisms in Awassi sheep, these genes can be considered important genetic markers. They can be used as markers in genetic improvement programs to improve sheep breeds' growth traits.

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