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Next Generation Sequencing (NGS) based variation analysis: A new practical biomarker for beef tenderness assessment

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Abstract

Evaluation of some meat quality attributes using genetic analysis is steadily increasing. PCR based targeted variation analysis is one of the most commonly preferred techniques for this purpose. Recently, Next Generation Sequencing (NGS) method has drawn considerable attention because of its' high analysis capacity. The purpose of the current study was to determine variations in CAST gene from Brangus and Simmental cattle by performing whole gene sequencing using NGS, and to investigate the potential of NGS method in evaluating meat tenderness based on the high genomic data it provides. Whole gene sequence analysis was performed on Calpastatin (CAST) gene of samples acquired from 52 Brangus and 52 Simmental beef cattle breeds using NGS method, and the variations detected were evaluated in terms of their potential in measuring meat tenderness and quality. NGS outputs were analyzed in Ensemble "cow" database platform and 13 variations were detected. One of these variations (EXON 8 c.439C>G/p.L147LV) was evaluated as undeclared before. In 20 Brangus cattle and in 9 Simmental cattle, no variations were detected whereas 6 variations (V1, V2, V5, V8, V10 and V13) were found significantly different ($p<0.05$) based on their distribution in breeds. Bearing in mind the developments in bioinformatics and NGS method which provides high volume of genomic data, use of these methods in evaluating tenderness of meat was thought to be more practical than assessment based on sensory analyses and instrumental texture evaluations.

Keywords: Beef tenderness, Polymorphisms, Calpastatin (CAST) gene, Next Generation Sequencing (NGS)

Introduction

Flavor, juiciness, and tenderness are very essential attributes by meat consumers (Aaslyng and Meinert, 2017). The taste and quality of meat is effected by the animals' species, age, kind of nutrition, sex, muscle type and environmental conditions (Klont, Brocks, and Eikelenboom, 1998; Wheeler, Shackelford, and Koohmaraie, 2000). Quality assessment of these characteristics can only be obtained after slaughter using classic conventional tests (Lu et al., 2013). Today's meat industry is generally using traditional phenotypic characteristics for this purpose. At the same time, the information obtained from extended genomic variations and genetic markers have opened up a new path for animal breeding and selection to supply feed and growth efficiency and improved carcass quality (Mateescu, Garrick, and Reecy, 2017). Several studies have shown that the genetic constitution (genotype) has significant effect on meat texture (Gao, Zhang, Hu, and Li, 2007; Van Eenennaam et al., 2007). Though several genes impact the

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meat quality, two genes in particular, Calpain (CALP) and Calpastanin (CAST), are directly associated with tenderness (Page et al., 2004; Barendse, 2002). The proteolytic enzyme system responsible for meat tenderness during postmortem aging is the calcium-dependent proteolytic system which includes three different constituents, m-calpain (CAPN1), μ-calpain (CAPN2) and a specific calpain inhibitor, calpastatin (CAST). Calpain activity is influenced by calpastatin which is the endogenous specific inhibitor for protease μ-calpain. Increased post-mortem calpastatin activity is correlated with reduced meat tenderness. Variations in calpastatin gene change the activity of the gene and affect the tenderness of postmortem meat (Zhou et al., 2017; Bhat, Morton, Mason, and Bekhit, 2018; Leal-Gutiérrez and Mateescu, 2019; Herrera-Mendez, Becila, Boudjellal, and Ouali, 2006; Enriquez-Valencia et al., 2017; Curi et al., 2009).

The genomic sequence of bovine CAST contains 35 exons spanning nearly 130 kb (Raynaud et al., 2005). Based on amino acid sequences, six different domains can be recognized some of which have been reported to be involved in binding calpastatin to biological membranes having a central role in the regulation of Ca^{2+} channels (Djadid, Nikmard, Zakeri, and Gholizadeh, 2011).

Targeted variation analysis has been used as a marker and detection tool to estimate meat quality traits including tenderness. One of the classical DNA methods, polymerase chain reaction (PCR) with targeted variation analysis techniques is applied for this purpose. This method serves to detect targeted variations by analyzing a restricted gene or the region of a gene. Associations between the genetic variations in relation to these components and meat tenderness have been determined in different meat types. Parra-Bracamonte, Martinez-Gonzales, Sifuentes-Rincon, and Ortega-Rivas (2015) detected the frequency of alleles of genetic markers related to tenderness in meat obtained from five Zebu breeds of beef cattle in Mexico and noted that there is a strong association between tenderness, and prevalence and distribution of polymorphisms in calpain and calpastatin. Zhou et al. (2017) investigated the associations of single nucleotide polymorphisms (SNPs) in CAPN1 and CAST genes from six generations broilers with some carcass characteristics and tenderness indicating that while in CAPN1 gene, SNP1, SNP2, SNP3 and SNP4 showed an association with carcass and tenderness characteristics, for CAST gene, SNP5 and SNP6 might be potential candidates as molecular markers for molecular assisted selection.

In recent years, next generation sequencing (NGS) technology has become a powerful tool as an accurate and rapid method in concurrent analysis of large amounts of genes or large DNA regions, and also in detection of new mutations or variations as well as noted variations. NGS technology has emerged as a promising approach for rapid detection of economically-motivated adulteration in meat mixtures (Cavin, Cotteneta, Cooperb, and Zbinden, 2018). However, the use of NGS technology in meat science and technology is very limited. NGS technology can provide abundant data thanks to its' high analysis capacity. However, analysis of this data and relevant bioinformatic studies are yet to keep up with the data provided by the method (Goodwin, Mcpherson, and Mccombie, 2016). NGS technology can provide reliable results as it has high reading capacity. However, compared to previous methods, due to high amount of DNA sequence data, high number of variations and errors are detected. On the other hand, variations detected by NGS must be confirmed using Sanger sequencing (Sanger, Nicklen, and Coulson, 1977). Analytic capacity and reliability of the two tests are certainly different; in targeted variation analysis, Sanger and NGS methods are equally reliable. However, if they are compared in a study concerning the sequence analysis of a larger region, Sanger sequencing is more reliable than NGS. As a matter of fact, in whole exon sequencing or whole genome sequence screening, analytic validation of NGS decreases and its' concordance and sensitivity rates drop to 95-97%. This is caused by the decline in reading depth, presence of artifact variants, repeat sequences and wide constitutional variants. Targeted Sanger sequencing panels provide higher coverage with a shorter test run time, and data set handling takes less time than NGS applications (Linderman et al., 2014; De Koning, Jongbloed, Sikkema-Raddatzand, and Sinke, 2014). As the NGS application is increasing, the acquired data will increase the bioinformatic analysis capabilities and due to its sensitivity, specificity and accuracy rates of the test will also increase. NGS technology allows us to include more genomic data and more variations in the test simply by using a nasal swab or blood sample. Because of this, when compared to other classic methods (based on sensory perception and instrumental texture analysis), it is much more practical and can provide faster results.

The purpose of the current study was to determine variations in CAST gene from Brangus and Simmental cattle slaughtered in Turkey by performing whole gene sequencing using NGS technology, instead of PCR based targeted variation analysis. Another purpose of the study was to research the potential of using high genomic data provided by NGS method

for evaluation of meat quality, particularly tenderness.

Materials and Methods

Sample Collection

Whole gene sequence analysis was performed on Calpastatin (CAST) gene on samples acquired from 52 Brangus and 52 Simmental beef cattle breeds using NGS method and the variations detected were evaluated in terms of their potential in measuring meat tenderness and quality. 52 Brangus breed cattle were provided from "Sakarya Meat Processing Plant of General Directory of Meat and Milk Board, Sakarya" and 52 Simmental breed cattle were provided from "Sincan Meat Industry Plant of General Directory of Meat and Milk Board, Ankara". The animals were 16 months old on average, weighing at least 450 kg male cattles. Two meat samples of 1x1x1 cm thickness were taken from the carcass of each animal, labeled, and stored at -20 ºC in Eppendorf tubes for further genetic analyses.

DNA isolation

The samples collected from both breeds and stored at -20 ^{0}C were collectively put through DNA isolation procedure. The meat samples were homogenized by being cut into pieces using a scalpel. Later on, DNA isolation procedures took place using QIAamp DNA FFPE Tissue Kit (50) (Qiagen). After isolation, concentration of DNA samples was measured and archived.

Spectrophotometric Measurement

The amount and concentration measurement of isolated DNA samples were performed by "NanoDrop Spectrophotometry".

PCR amplification and Gel Electrophoresis

Before NGS analysis, multiplex PCR method was used to amplify the target regions of the sample DNA. A Primer coupled with an adapter designed to encompass the exon-intron binding areas and the 31 exons of the CAST gene was used for this purpose. Owing to the primers with adapters, samples and regions could be differentiated (Table 1).

Table 1. 31 exons and exon-intron binding areas of the CAST gene with appropriate adapter primers

Region	Primer (forward)	Region	Primer (reverse)
CASTex1-F	GCCCTCGCTCCCTCCCAG	CASTex1-R	CCGGTCACCTGCCCAGAG
CASTex2-F	CCTATGTCAATGGAGAATTATTAACAGTTC	CASTex2-R	GGAGATATTTGCTACCTCATTATTTATTTCAT
CASTex3-F	TGCGGTTGACCACACTGTTAAG	CASTex3-R	TGCCCAGAAATGATACTTTGTTCCA
CASTex4-F	TCAGCCACGATTGAGTGACTAAC	CASTex4-R	AATCCTGTATAAGTATATAGATGGTGTTTGAG
CASTex5-F	TGTTAATTCGTGTTGCTTACTTGACT	CASTex5-R	AGCGTTACAGAAGATGGTGAACT
CASTex6-F	AAAGCATAATAATCTTAACTCACAACACT	CASTex6-R	AGCTATTCATTATTATTTCAAAGAATCCCA
CASTex7-F	AACCAGACACCAACAGCCATT	CASTex7-R	AATAACTGCCATTCTAGGTAGGACTT
CASTex8-F	AAGTGTTATGAATTGCTTTCTACTCCTC	CASTex8-R	TCATCTGTCTGCTTTATTTACCTTTGG
CASTex9-F	GCTAGTGACCATTTCCCTACAAGAT	CASTex9-R	GACGCACGCTCCTCTTCATC
CASTex10-F	TATCATTGTTATTATTACTTCTGCTGTTCTG	CASTex10-R	TTAAGCTAACCTCACCTCATATTGTT
CASTex11-F	AAGTGAAGGATGTGCAGCAAGTA	CASTex11-R	GCTACCACGGACGCTAACAG
CASTex12-F	ACTGCTGGCTTCTTAATGATTTGTAT	CASTex12-R	CCATCCAATCTGTAACACTCTGAC
CASTex13-F	ACACGACTGAGCGACTGAACT	CASTex13-R	CCCACCCTCTTCCTTTGAATAGATG
CASTex14-F	AATCTGTTCTGTCACTTAAATGGTTCC	CASTex14-R	AGCCTACACATCGCAACTAGAGA
CASTex15-F	TATGTTTCCTTCATCTGCCAGTCAA	CASTex15-R	GAGGTCTACGGGTATAATGCACTATT
CASTex16-F	GCCACAGCTCATTCCTAGAGATT	CASTex16-R	TATGTTGGGCATTAGTTTCGTAACC
CASTex17-F	TTCCTCCACCTCCAGTCTCC	CASTex17-R	TTCAGGGTTTCCAGAGTTGTTATCT
CASTex18-F	AATACAAACTCACTCAAACATATCAGAAA	CASTex18-R	TTCTCCTTAATACTAGGCTGGCATAT
CASTex19-F	ATTCATTACTTGTTGTGTGACATTTATCT	CASTex19-R	AATACGTTTGGTCCTGGCATTT
CASTex20-F	CCGTATTGTTGGTTCATTGTTGTC	CASTex20-R	GTAATACATTGGTAATACAGGAGGAAGG
CASTex21-F	TCTGAGTTGTTCGTTGTAGTCTCTT	CASTex21-R	CGCTCGCTCTGCTTCACTT
CASTex22-F	TTATCAGAGAACGAGGTACTAACACT	CASTex22-R	TGCTAACAGGATGTGAGTTAAGTAATAC
CASTex23-F	ATCATCAGCTATAACCTATCAACCTCT	CASTex23-R	TCTGCCCTTCCTAAATTAACCATCA
CASTex24-F	CGAGGTAGCGTTTGCTGACA	CASTex24-R	CACTGGCTCTATTAGTTACACTGTTG
CASTex25-F	TGCTGTGTTGCTGTGCTTCC	CASTex25-R	CCTATCTTGCCAGTCTTACCTCTTC
CASTex26-F	CTAAGGTGGCTAACCAGTGACTAAT	CASTex26-R	TCTCTGTTCGTTTCCAAGGCAAA
CASTex27-F	TCTGTTGACATTGTTGCTCTAAGTTAC	CASTex27-R	GAGTATAGATCCAACCTGGACACC
CASTex28-F	ACTGGATAAAGATCATGTAAATACTGACTTA	CASTex28-R	GCACAAGGTAGGCATTCACTGA
CASTex28-F	CTCAGCACCTTGTATAACAGAGTG	CASTex28-R	AAGTTTCCTAGGGCATTAATCAGTTTA
CASTex30-F	AGTTAATTGCTAGATGGAGTGTTGAC	CASTex30-R	TGGAGAAGGAAATGGCAACCC
CASTex31-F	GGGAAGAATTCAGTGTTTGGACTAAA	CASTex31-R	AGATTTCAGTGTCCCTTTCATTGC

Afterwards, a purification process was performed using magnetic beads. Then, DNA fragments containing 31 exons, identified as specific bands in 2% agarose gel electrophoresis were observed and inspected. Following this, multiplex PCR products were registered and stored at -20°C to be used in NGS screening.

Next Generation Sequencing (NGS) Analysis

Following the proliferation of DNA samples using adapter primers and the purification procedure, library preparation steps were applied. During the library preparation, Nextera V2 kit (Illumina, California) was used; afterwards, samples were uploaded to the NGS device (Miseq-Illumina, USA). NGS outputs were analyzed in the Ensembl data base. The detected variations' pathogenity was evaluated in the databases and outputs were recorded for each animal.

Interpretation of (NGS) Results

In the genome of every organism along with some common sequences there are several different sequences (variations). If there is a change especially in the coding sequences in functional regions of the gene (variations) that causes a malignant manifestation (that is, causing a change in the amino acid sequence and protein conformation), this change is referred to as "pathogen variation" (mutation) (Linderman et al., 2014). On the other hand, benign variations or polymorphisms can lead to favorable or unfavorable results (Richards et al., 2015). Whether a variation is pathogenic or not depends on factors such as its' localization (intronic, exonic or splice site), changes expression or post-translational defects and ultimately its frequency. The probability of a variation being pathogenic increases if it is Minor Allele Frequency (MAF) value is under 1%, whereas a frequency rate above 1% it is likely to be a polymorphism (Richards et al., 2015). Other determining factors for pathogenic variation are; along with the frequency of the change, its' localization (intronic or exonic); being a nonsense, frameshift change or being localized in start codon or it is a change concerning the deletions of a few exons. On the other hand, the definition of variation in different literatures and how it has been described by different researchers (pathogen/ polymorphism) is also an important criterion and it is possible to understand if the variation in question is a pathogen by referring to different databases (Linderman et al., 2014).

In today's world, several and very rich databases and analysis programs have been developed. There are also databases and analysis programs concerning plants and different animal species. These programs compare the reference sequence with the target region. One of these databases, Ensembl, is a platform that includes the DNA sequence information of cattle genome under the name "cow" (Ensemble Cow Database, 2019).

In this study, we performed the comparison and analysis of the DNA outputs we obtained using NGS analysis with the Ensemble "cow" cattle genome with the "mutation survayor" program (1-5 Ensembl web). Before the analysis, the quality scores of NGS raw data (such as amount of DNA and reading values) were checked using the Mutation Surveyor.

Results and Discussion

CAST gene has been proven to have an impact on the tenderness and textural features of the meat following the slaughter of the cattle (Yousefi and Azari, 2012). In this study, by using NGS method, the correlation between the CAST gene and meat tenderness was not only explained through polymorphic variations but also provided a chance to detect all possible polymorphisms. Thus, a dynamic and practical method based on genetic markers for meat cattle farming for breed or animal preferences was targeted. As a result of the analysis, 13 variations were determined. Unlike the Sanger sequencing and target polymorphism studies that were used in many previous surveys, a dynamic analysis was performed in this study. With the NGS technology, more variations were determined and included in the study and also new variations were identified (Table 2). The NGS outputs were analyzed in the Ensembl database.

When the analysis outputs are taken under consideration, one (EXZON 8 c.439C>G/ p.L147LV) of the 13 polymorphisms we detected is a variation that has not been reported before. One of the polymorphisms we detected (EXZON 22 c.1632A>G/p.E544E) is a splice region variation and therefore, in silico indicates "likely pathogenic" quality. This polymorphism has been detected in both Brangus and Simmental cattle breeds and observed that it has no pathogenic phenotypical effect. The c.439C>G/ p.L147LV variation was found only in 2 samples (numbered 9 and 26) of Brangus breed and was not observed in any cattle of Simmental breed. If the polymorphisms we detected and indicated in Table 8 are taken under consideration in terms of their frequency, all of them are above 1% (average frequency 25.55%) except three (EXON 20 c.1510C>T / p.P504S, INTRON 6 c.373-3C>CT and EXON 8 c.439C>G/ p.L147LV) qualify as polymorphisms. In 20 animals in Brangus group, and 9 animals in Simmental group, no variations were detected. The presence of variations and the group correlations were examined using cross tables and fisher exact test. (Table 3). Based on this; there is a statistically significant correlation ($p<0.05$) between the presence of V1, V2,

V5, V8, V10 and V13 variations and the study groups (Table 3).

V1 variation (EXON 20 c.1526T>C/p.V509A) was observed in 48.1% of Brangus cattle while 82.7% in Simmental cattle group (p=0.001). V2 variation (EXON 22 c.1632A>G/p.E544E) was detected in 30,8% of Brangus cattle breed whereas 80.82% in Simmental cattle group (p=0.000). V5 variation (EXON 9 c.616G>A/p.E206K) was observed in 13.5% of Brangus breed, and 36.52% of Simmental group (p=0.006). V8 variation (EXZON 26 c.1985G>C/p.S662T) was not observed in Brangus group while it was found as 21.2% in Simmental group (p=0.000). V10 variation (EXON13 c.895 G>A/p.A299T) was observed in %5 of Brangus group and not at all in Simmental group (p=0.028). While V13 variation (INTRON 18 c.1335+6G>A) was not detected in Brangus group, in Simmental group it was observed as 25% (p=0.000).

Table 2. Variations and their Minor Allele Frequency (MAF) values

#	VARIATIONS	RS NUMBER	MAF (Ensembl)	MAF (Current Study)
V1	EXON 20 c.1526T>C/p.V509A	rs109384915	38%	42.78%
V2	EXON 22 c.1632A>G/p.E544E	rs110712559	25%	36.53%
V3	EXON 14 c.934A>G/p.N312D	rs723916435	23%	1.92%
V4	EXON 9 c.583A>G/ p.T195A	rs210072660	44%	25.00%
V5	EXON 9 c.616G>A/p.E206K	rs384020496	19%	2.50%
V6	EXON 8 c.439C>G/ p.L147LV	undeclared	-	0.96 %
V7	INTRON 22 c.1714-3C>T	rs110711318	21%	2.88 %
V8	EXON 26 c.1985G>C/p.S662T	rs110914810	45%	8.65%
V9	EXON 9 c.630G>AG/ p.K210KK	rs378682309	15%	1.92%
V10	EXON13 c.895 G>A/ p.A299T	rs715323791	-	2.40%
V11	EXON 20 c.1510C>T / p.P504S	rs1116977475	-	0.48%
V12	INTRON 6 c.373-3C>CT	rs433558933	-	0.48 %
V13	INTRON 18 c.1335+6G>A	undeclared	-	7.69%

Table 3. Frequencies and level of significance for variations detected in Brangus and Simmental beef cattle

Variation	Presence	Brangus n	Brangus %	Simmental n	Simmental %	P Value
V1	No	25	48.1	9	17.3	0.001*
	Yes	27	51.9	43	82.7	
V2	No	35	67.3	10	19.2	0.000*
	Yes	17	32.7	42	80.8	
V3	No	48	92.3	52	100.0	0.059
	Yes	4	7.7	0	0.0	
V4	No	36	69.2	29	55.8	0.112
	Yes	16	30.8	23	44.2	
V5	No	45	86.5	33	63.5	0.006*
	Yes	7	13.5	19	36.5	
V6	No	50	96.2	52	100.0	0.248
	Yes	2	3.8	0	0.0	
V7	No	51	98.1	47	90.4	0.102
	Yes	1	1.9	5	9.6	
V8	No	52	100.0	41	78.8	0.000*
	Yes	0	0.0	11	21.2	
V9	No	52	100.0	50	96.2	0.248
	Yes	0	0.0	2	3.8	
V10	No	47	90.4	52	100.0	0.028*
	Yes	5	9.6	0	0.0	
V11	No	51	98.1	52	100.0	0.500
	Yes	1	1.9	0	0.0	
V12	No	51	98.1	52	100.0	0.500
	Yes	1	1.9	0	0.0	
V13	No	52	100.0	38	73.1	0.000*
	Yes	0	0.0	14	26.9	

* $p<0.05$

Conclusion

Among the factors that affect the flavor and tenderness of beef that are as significant as genetic factors are the geography of where the cattle are farmed, feeding and environmental factors. However, when the undeniable effect of genetic factors is taken under consideration, genetic based evaluation tools are an efficient method for beef or animal selection. The main purpose of these kinds of studies is that more genetic biomarkers (variations) are scanned with more practical and reliable methods, and the acquired data are analyzed to generate genotype and phenotype profiles. NGS technology provides an important potential in achieving this purpose thanks to its high analysis capacity.

This study focused on NGS based genetic profiling as a more tangible and practical approach compared to sensory perception and textural based methods. The CAST gene variation profiles of highly preferred Simmental and Brangus cattle breeds in our country were compared using NGS method and it was found that the difference in distribution of variations among the two breeds is statistically significant. In upcoming studies, the genetic variation profiles and the sensory and instrumental texture evaluations can be compared to find out the phenotypical outcome of the detected variations. Therefore, NGS technology would become a very practical and comprehensive technique in evaluating meat quality and tenderness. Over time the increase in these kinds of applications will enrich the current genomic data, increase the sharpness of bioinformatic analysis and the technique will eventually be completely validated. This method is DNA based and can be applied using any biological material (blood, nasal mucosa etc.) unlike sense perception and textural analysis methods. Thus, in practice it can be applied on live animals and allow adequate animal selection for slaughter.

NGS is a strong candidate considering the anticipated developments in bioinformatics in the near future. One of the advantages of preferring NGS method for evaluating the tenderness of meat and animal selection is that it can scan several gene loci and variations, and these data can be used as selection criteria. Over time, with the increase in genomic data, the precision of the test will increase even more. By analyzing NGS outputs with advanced analysis programs, not only can the already identified variations be defined but also new variations could be identified as pathogenic based on in silico parameters.

The method based on DNA analysis and the bioinformatic analysis of several variations is a validated, more reliable method when compared to other methods such as sensory evaluation which are subjective and not standardized and can lead to difficulties in their application (the education of the participants who will evaluate, subjective evaluation criteria, etc.). When all these advantages are taken under consideration, use of NGS technology will become more commonly preferred in selecting quality meat for consumption.

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