Original Article

Single Nucleotide Polymorphism (SNP) of leptin gene in holstein cattle

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Abstract

Leptin is a versatile important hormone that regulates several metabolic pathways, such as controlling body weight, feed intake, energy expenditure, immune function, and reproduction. This study was conducted to describe the single nucleotide polymorphisms (SNPs) within bovine leptin (*LEP*) gene, and their possible consequences on leptin structure and function in Holstein cattle. After collecting blood samples from 60 Holstein cattle, extracting their genomic DNA, two PCR-specific primers were designed. Both PCR and SSCP experiments were optimized and performed for each PCR fragment. Each different set of SSCP resolved bands was sequenced and analyzed. Two SSCP patterns representing two genotypes (AA, and AB) were detected with two alleles. Four SNPs were observed in this study, intronic, exonic synonymous, exonic non-synonymous, and nonsense SNP. All the observed SNPs were computationally analyzed using several bioinformatics tools. The most exciting novel SNP was a p.E115Ter nonsense mutation, which caused a premature termination of leptin and truncation of its last thirty-one amino acid residues. The deleted segment contained α-helix E, α-helix D, and Cyst146. Thus, it is strongly suggested that such mutation has a series of deleterious consequences, at least in terms of leptin receptor binding or its subsequent signaling activity. Nonetheless, such highly damaging mutation might not be abolished for the whole milk production trait in the studied cattle population. Although this study did not associate the observed mutations with production traits, it shows the findings of dramatic nonsense mutation, which might be important in future studies, and laid the basis for further association analyses needed to evaluate the potential use of this truncation as genetic hallmarks of the highly economically important Holstein cattle breed.

Keywords: Holstein, leptin, milk, nonsense mutation, sequencing, SNPs

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Introduction

Improvement in production traits and increase in economic gain of the population are the main goals of all cattle breeders. In addition to relying on the traditional selection program, breeders utilize genetic markers to accomplish this task. The latter strategy provides potentially powerful tools for identifying the favorite breed of dairy cattle (Felius et al., 2015). The implications of genetic tools in Holstein cattle have been found to effectively produce a rapid economic gain for the intended industrial goals (Zenger et al., 2006). Several candidate markers have been targeted on the level of DNA, one of these markers is the *LEP* gene (Yoon et al., 2005). It plays a crucial role in the regulation of feed and energy metabolism of the body (Liefers et al., 2005). The *LEP* gene encodes a hormone that regulates body weight by maintaining the balance between food intake and energy expenditure through signaling to the brain to change the stored energy levels (Zhou et al., 2009). In addition to the obvious role of *LEP* gene in controlling appetite, it has other roles in regulating growth, reproduction, body composition and immunity; therefore, the *LEP* gene may be a strong candidate for evaluation of genetic polymorphism (Chamberlain et al., 2010). Accordingly, there is necessity for an easy to use tool to detect the unknown SNPs of this highly important gene. Several techniques have been employed to exploit the portions of this gene to detect the extent of polymorphisms, one of them is single stranded conformation polymorphism (SSCP), which is characterized by its ability to detect the unknown mutations (Gasser et al., 2006). Indeed, the utilization for SSCP polymorphisms could lead to the findings of new useful genetic markers of agricultural populations (Yoon et al., 2005). The *LEP* gene could be considered as one of the best interesting biological markers in cattle since it possibly plays a significant role in "marker assisted selection" as it is positioned on the 4q32 position of the bovine chromosome (Agarwal et al., 2009). The size of *LEP* gene is estimated to be about 20 kb and it consists of three exons separated by two introns, and the exons for the *LEP* gene cover about 15 kb of the genome. Actually, the first exon is not transcribed; instead, the remaining two exons produce the whole 167 residues of leptin. Then, the first 21 signal amino acid residues of leptin were excised to produce the mature 16 KD blood circulated protein (Liefers, 2004). Leptin contains a distinctive three-dimensional four-α-helix bundle structure (Kline et al., 1997). In addition, a nuclear magnetic resonance analysis of a crystalline form of leptin revealed that the leptin four-helix protein A-B-C-D pattern was similar to the structure of the cytokine family (Zhang et al., 1997). This structure is arranged in a four sequentially similar anti-parallel left-hand twisted α-helices bundle that is connected by two crossover links, along with one short loop (Gutierrez et al., 2009). In addition to the four main helices, an extra fragment, a distorted short helix called helix E, is also presented on the structure. The helix E is found in the loop linking between both helices C and D. Actually, helix E has made leptin molecule distinct from the high structurally close class I helical cytokines (Zhang et al., 1994). Leptin contains a single disulfide bond that links two cysteine residues (Cys96 and Cys146) within the C and D helices to form a unique kink. This kinked helix, which connects the last turn of the α-helix D to a loop that extends from the C to D helix, has been proven to be very crucial for the structural integrity and stability of leptin (Rock et al., 1996). Hence, any SNP that changes this highly organized three-dimensional structure of leptin may produce deleterious consequences on many critical metabolic pathways this hormone is involved in. On the other hand, it was obviously demonstrated that many SNPs had functional effects on their corresponding protein structure either by a single change in the amino acid (Bahrami et al., 2013) or by modulation of the transcription regulation rate (Liao and Lee, 2010), or by other reasons. Synonymous mutations - sometimes called 'silent' mutations - are now widely acknowledged to be able to cause changes in protein expression, conformation, and function (Sauna and Kimchi-Sarfaty, 2011). However, mutations in introns and other non-coding regions do not alter amino acid sequences. Conversely, a non-synonymous single nucleotide polymorphism (nsSNP), which is present within the exon of a gene, is responsible for the incorporation of an alternative amino acid and known to be one of the main causes of the possible alteration of the leptin mode of action. However, tolerant nsSNPs are not deleterious and are not involved in any changes, whereas deleterious nsSNPs have a profound influence on protein structure and its interaction. Therefore, it is important to computationally differentiate deleterious nsSNPs from tolerant nsSNPs. Although several SNPs of the *LEP* gene have been identified and associated with several functional productive characters, such as milk yield and composition (Glantz et al., 2012), performance (Giambra et al., 2014), and production in Holstein cows (Clempson et al., 2011), the number of researchers that dealt with the effects of these SNPs on the three-dimensional structure have not been studied extensively. This potential association must be predicted using several up-to-date analysis tools. Therefore, sequential genotyping experiments on the *LEP* gene using the PCR-SSCP-sequencing *in vitro* method were performed in this study to identify its potential unknown SNP(s) in Holstein cattle. Then, several *in silico* tools were used to analyze the consequences of each observed SNP on both structure and function of leptin.

Materials and Methods

Blood sampling and DNA extraction: The study was conducted in accordance with the international recommendations for the care and use of animals and both maintenance and feeding were similar for all animals and remained in accordance with proper animal welfare guidelines for the care and use of dairy cattle (Federation of Animal Science Societies, 2010). About 3 to 4 ml of blood samples were collected from the jugular veins of 60 Holstein cattle and placed in anticoagulation tubes. Genomic DNA was isolated using a manual extraction method (Al-Shuhaib, 2017). The extracted DNA was evaluated using a Nanodrop (BioDrop μLITE, Biodrop, UK).

PCR analysis: Two pairs of primers were designed to partially cover intron 1, exon 2, intron 2, and exon 3 of the cattle *LEP* gene using the NCBI primer BLAST online software (Table 1). PCR reaction was performed using AccuPower PCR premix (Cat # K-2012, Bioneer, Daejeon, South Korea). The amplification was begun by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C (for 291 bp product), or 62°C (for 290 bp products) for 30 s, and elongation at 72°C for 30 s, and was concluded with a final extension at 72°C for 5 min.

Table 1 Designed primers for both exon 2 and exon 3 of *LEP* gene

Genbank accession no.	Sequence (5′-3′)	Length (bp)	Amplified segment
KY245895	GGATGCGGGTGGTAACGGAGCAC CGGGATGGCCACGGTTCTACCTC	293 bp	partially cover intron 1, exon 2, and intron 2
KY245896	CAATGACCTGGAGAACCTC ACATAGGCTCTCTTCTCCTGT	290 bp	partially cover exon 3

SSCP analysis: SSCP experiments were performed according to the protocol of Al-Shuhaib et al. (2017). The gel was run at constant conditions (200 V/100 mA/100 min) at room temperature and stained by PAGE red gel dye (Cat # 41014, Biotium, Hayward, USA).

Statistical analysis using PopGene32: Statistical analysis of genetic diversity was performed for each genetic fragment individually to estimate allele and genotype frequencies, as well as Nei's heterozygosity. These criteria were calculated with PopGene32 software, v; 1.31 (Yeh et al., 1999). A χ^2 test was performed to verify possible deviations from Hardy-Weinberg equilibrium (HW) expectations for the distribution of genotypes. Average heterozygosity was employed to estimate genetic diversity within the population.

DNA sequencing and sequencing analysis: A template DNA sample that showed a different pattern on the SSCP gel, of AB and AA genotypes for both PCR amplicons, was sequenced from both ends (Macrogen Inc. Geumchen, Seoul, South Korea). The cattle reference database (GenBank Acc. No. AC_000161.1), together with its exon 3 position, was retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov). Sequencing results of the PCR products of different SSCP patterns were edited, aligned, and analyzed together with the respective sequences in the reference database using *BioEdit* Software Ver. 7.1 (DNASTAR, Madison, WI, USA). The two observed genotypes were translated into amino acids in a reading frame corresponding to the reference leptin amino acid sequences using the *Expasy* online program (http://web.expasy.org/translate/). Multiple amino acid sequence alignment was made between the reference exon 3/*LEP* amino acid sequences and its two observed genotypes using the *UniProtKB* online software. Then, the sequencing results of the PCR products for Lep studied loci were submitted into NCBI-bankIt server (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) and assigned Genbank accession numbers (GenBank: KY245895.1 and GenBank: KY245896.1 for 393 bp and 390 bp, respectively).

Prediction of nuclear receptor binding site for leptin using NHR-scan: NHR-scan is a computational predictor of nuclear hormone receptor binding site's web server (http://cisreg.cmmt.ubc.ca/cgi-bin/NHR-scan/nhr_scan.cgi). It can predict and classify potential nuclear hormone receptor binding sites in genomic sequences using Hidden Markov Models (HMM) derived from a large collection of verified binding sites (Sandelin and Wasserman, 2005). Essentially the NHR-scan model consists of three 'match state chains' - corresponding to each type of site configuration (direct repeat or DR, inverted repeats or IR, and everted repeats or ER), and one 'background state' - corresponding to no prediction.

Prediction of noncoding SNP effect on splicing events using ESEfinder: ESEfinder (http://exon.cshl.edu/ESE/) analyzes exonic sequences to predict putative exonic splicing enhancer (ESE) (Cartegni et al., 2003). ESEfinder uses weight matrices to identify putative ESE in query sequences. The matrices are based on frequency values obtained by the functional systematic evolution of ligands by exponential enrichment (SELEX) experiments and the initial SELEX library, which was made by chemical synthesis. Only noncoding SNPs were involved in this prediction tool.

Findings of deleterious effect of LEP nsSNP using SIFT: The amino acid sequence of *LEP* along with non-synonymous SNPs (nsSNPs) with corresponding amino acid positions were submitted using the SIFT (sorting intolerant from tolerant) program (Pauline et al., 2003). The SIFT prediction was given as a tolerance index (TI) score ranging from 0.0 to 1.0. Substitutions at each position with normalized probabilities less than a tolerance index of 0.05 were predicted to be intolerant or deleterious; those greater than or equal to 0.05 were predicted to be tolerated (http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html).

Validation of functional characterization of LEP nsSNP using PANTHER: The SIFT predicted nsSNPs were validated by the *PANTHER* (protein analysis through evolutionary relationship) program (Tang and Thomas, 2016), which is an open access server that estimates the likelihood of a particular nsSNP to cause a functional impact on the protein (http://www.pantherdb. org/ tools/csnp). PSEP (position-specific evolutionary preservation) measures the length of time (in millions of years) a position in the current protein has been preserved by tracing back to its reconstructed direct ancestors. The longer a position has been preserved, the higher the functional impact a particular

amino acid substitution is likely to have. The thresholds chosen were: "probably damaging" (time > 450 my), "possibly damaging" (450 my > time > 200 my, corresponding to a false positive rate of ~0.4) and "probably benign" (time < 200 my).

Investigation into mutant protein stability of LEP nsSNP using I-Mutant 2.0: To have a better insight into the stability of the protein caused by mutation, mutated positions were analyzed using I-Mutant 2.0 (Capriotti et al., 2005). I-Mutant 2.0 (http://folding.biofold.org/cgi-bin/i-mutant2.0) is a Support Vector Machine-based web server for the automatic prediction of protein stability changes upon single-site mutations. The input FASTA sequence of protein along with the residue change was provided for analysis of DDG value (kcal/mol). Also, the RI value (reliability index) was computed.

Prediction of severity effect of LEP nsSNP using SNAP2: SNAP2 is based on a machine learning device called "neural network." It distinguishes between effect and neutral variants/ nonsynonymous SNPs by taking a variety of sequence and variant features into account (Smigielski et al., 2000). Predicting a score (ranges from −100 strong neutral prediction to +100 strong effect prediction), the analysis suggests that the prediction score to some extent correlates with the severity of effect (https://www.rostlab.org/services/SNAP/).

Prediction of functional impact of LEP nsSNP using PROVEAN: Further confirmation of the effect of nsSNPs on protein was done using the PROVEAN (Protein Variation Effect Analyzer) tool (Choi et al., 2012), which can predict the impact of an amino acid substitution on the biological function of a protein (http://provean.jcvi.org/index.php). This algorithm allows for the best-balanced separation between the deleterious and neutral amino acids, based on a threshold. The default threshold is -2.5, i.e. variants with a score equal to or below -2.5 are considered "deleterious", while variants with a score above -2.5 are considered "neutral".

Three-dimensional leptin structure modeling using RaptorX and PyMol: The whole amino acid sequences of bovine leptin were retrieved from NCBI (GenBank acc. No. AJ512638). The UniProtKB/Swiss-Prot accession number for bovine leptin is P50595 and no matching PDB entries were found in this protein (http://www.rcsb.org/pdb/protein/P50595?evtc=SuggestandevtaUniProtAccessionandevtl=autosearch_SearchBar_querySuggest). Out of 167 amino acids of the whole leptin, the first 21 amino acids that function as a signal peptide and are cleaved off before releasing the remaining 146 amino acid protein into the blood (Liefers, 2004) were omitted from the three-dimensional structure prediction. The three-dimensional structure of the *LEP* gene was constructed from the online three-dimensional model prediction software, RaptorX (http://raptorx.uchicago.edu/StructurePrediction/predict/). The observed mutations were inserted in the native sequence of the *LEP* protein to represent both AA and AB genotypes. Then, the three-dimensional structure of the native *LEP* protein, as well as AA and AB genotypes, were predicted using the RaptorX tool. The virtual proposed changes within its corresponding mutants were performed by using PyMOL-v1, 7.0.1 software (www.shrodinger.com), through which the distance of polar interactions with other amino acids was measured before and after mutation.

Construction of phylogenetic tree of truncated LEP using MEGA5.1: A phylogenetic tree was constructed for our resolved two genotypes to detect any distinctive feature among those from the original *Bos taurus* protein and other closely and non-closely related organisms. The Molecular Evolutionary Genetics Analysis (MEGA) software (http://www.megasoftware.net), which can provide a biologist-centric, integrated suite of tools for statistical analyses of DNA and protein sequence data from an evolutionary standpoint (Tamura et al., 2011).

Results

The polymorphisms of *LEP* gene was detected by the PCR-SSCP and DNA sequencing methods. Two specific PCR fragments were designed. The first one was designed to partially cover intron 1, completely cover exon 2, and partially cover intron 2. The second fragment was designed to partially cover exon 3. The observed variations of SSCP gels indicated the detection of two different genotypes with two alleles for both analyzed portions (Table 2).

Table 2 Observed, expected and heterozygocity, χ^2 test for Hardy-Weinberg equilibrium, and allelic frequencies of A) *DGAT*2 5'-UTR/ exon 1 fragment, and B) *BTN* 5'-UTR fragment for Holstein cattle breed

A) Intron 1/ Exon 2						
Obs-Het	**Exp-Het**	**Avr-Het**	**Nei-Exp -Het**	**χ^2**	**Allele A freq.**	**Allele B freq.**
0.2	0.2331	0.2311	0.2311	1.278	0.87	0.13
B) Exon 3						
Obs-Het	**Exp-Het**	**Avr-Het**	**Nei-Exp -Het**	**χ^2**	**Allele A freq.**	**Allele B freq.**
0.6	0.42	0.42	0.42	10.66	0.7	0.3

Abbreviations: Obs-Het – observed heterozygocity, Exp-Het – Expected heterozygocity, Avr-Het – Average -heterozygocity, Nei-Exp-Het – Nei's expected heterozygocity, freq. – frequency

Polymorphism analysis: The χ^2 test showed that the polymorphism of both portions in Holstein cattle was at Hardy-Weinberg equilibrium for these loci in the studied population. Nei's expected heterozygosity for

both genes in this study had the same value of average heterozygosity. This indicates high domination of allele A in both cases. In addition, the values of the observed heterozygosity for both genotypes in intron 1/ exon 2/ intron 2 fragments were higher than their expected values. This refers to the high level of genetic variability in the studied population regarding this fragment. The situation in exon 3 fragment was different from the values of the observed heterozygosity, which were equal to their expected values. For both studied loci, two genotypes, AA and AB, and two alleles, A and B, were observed (Fig. 1A and Fig. 2A). The observed frequencies of intron 1/ exon 2/ intron 2 fragment genotypes were 0.23 (n = 14) and 0.77 (n = 46) for the AB and AA genotypes, respectively. The PopGene32 program showed that the observed frequency of allele A was 0.87 and allele B was 0.13 (Table 2, A). In exon 3 fragment, the observed genotype frequencies were 0.6 (n = 36) and 0.4 (n = 24) for the AB and AA genotypes, respectively. The PopGene32 program showed that the observed frequency of allele A was 0.7 and allele B was 0. 3 (Table 2, B). Concerning intron 1/ exon 2/ intron 2 PCR fragments, the two distinct electrophoretic SSCP patterns were confirmed by sequencing experiments since one novel SNP detected discriminated between the two resolved genotypes and NCBI reference sequences (Fig. 1B). The pattern and nature of this SNP detected by sequencing indicated that the genotype AB had C instead of G in position 87 of this amplified fragment (Table 3A). However, this SNP was a noncoding and positioned in intron 1 of leptin since the start of exon 2 was triggered in position 106 of this PCR fragment (Fig. 1C). Thus, polymorphism and its consequent amino acid changes were not seen in the coding region of exon 2 in this cattle population (Fig. 1D). Concerning exon 3 PCR fragments, the two different SSCP patterns were confirmed by sequencing since three SNPs were detected between the two resolved genotypes. The pattern and nature of these observed SNPs indicated that both AA and AB genotypes have one unique SNP (Fig. 2B). In the AB genotype, a noncoding T>C SNP was observed in position 106 of PCR fragment, while a coding G>C SNP was observed in the AA genotype at position 94 of the PCR fragment (Fig. 2C). The latter SNP was a nsSNP, which yielded E>D amino acid variation at position 108 in the protein (Fig. 2D and Table 3B). Interestingly, a novel unexpected nonsense mutation was observed in both genotypes, in which Glu was substituted in Ter to cause a premature termination of protein at position 115 of the blood circulating leptin protein. Because of the latter SNP, a fragment of thirty-one amino acid residues was truncated from protein.

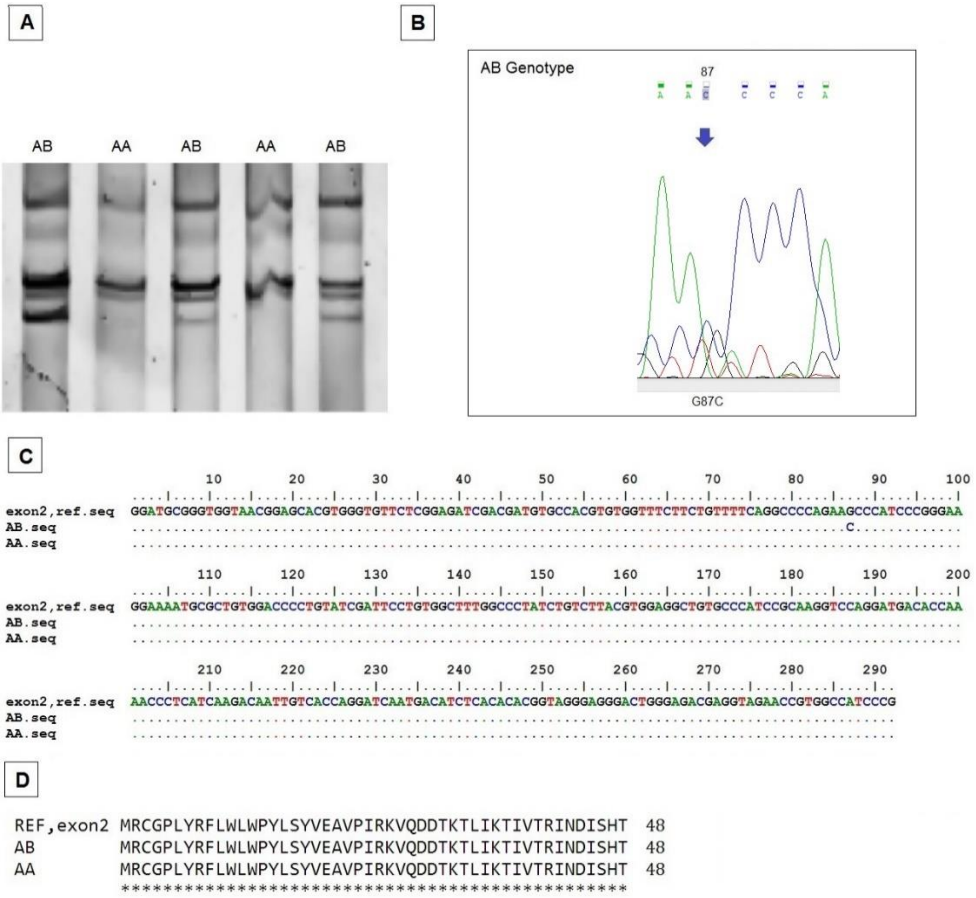

Figure 1 SSCP-sequencing results for Holstein *Bos taurus* partial intron 1/ exon 2 of the *LEP* genetic locus. **A**) PCR-SSCP gel profile of the two genotypes, AB and AA. **B**) Chromatogram pattern of G87C sSNP. **C)** Results of multiple nucleic acid sequences alignment of two SSCP genotypes with their reference sequence. **D**) Multiple sequence alignment of amino acid sequences that correspond to the coding region of PCR amplified segment.

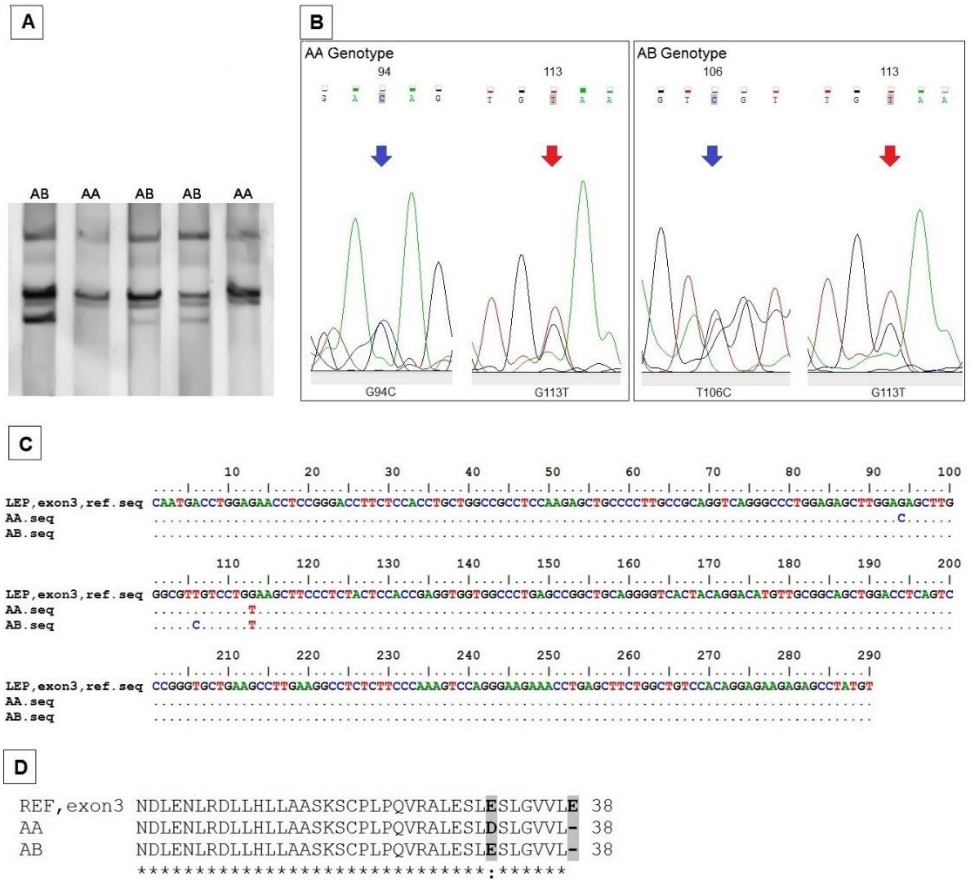

Figure 2 SSCP-sequencing results for Holstein *Bos taurus* partial exon 3 of the *LEP* genetic locus. **A**) PCR-SSCP gel profile of the two genotypes, AB and AA. **B**) Chromatogram pattern of G94C sSNP, T106C nsSNP, and G113T nonsense SNP. **C)** Results of multiple nucleic acid sequences alignment of two SSCP genotypes with their reference sequence. **D**) Multiple sequence alignment of amino acid sequences, the symbol "-" refers to the stop codon.

Table 3 List of all observed SNPs in both DGAT2 (A) and BTN genetic fragments (B)

Locus	Position	Genotype	Nucleotide substitution	Type of SNP	Amino acid change	Helix affected
A) intron 1	87	AB	G>C	Noncoding	Non	Non
	94	AA	G>C	nsSNP	E108D	α-helix E
B) exon 3	106	AB	T>C	sSNP	Non	Non
	113	AB and AA	G>T	Nonsense	E115Ter	α-helix D

Computational analysis: The possible effects of all observed SNPs on the binding of leptin with its nuclear receptors were analyzed using the NHR scanning tool. Regarding intron 1/ exon 2/ intron 2 PCR fragments, which included the observed novel intronic SNP, no binding was noticed, neither in the reference sequence nor in the observed sSNP of the AB genotype (Table 4A). The same non-effective role of the observed sSNP and nsSNP positions was observed in the exon 3 PCR fragment, while dramatic changes occurred in the position of the observed novel nonsense mutation in the exon 3 PCR fragment (Table 4, B). Among the four most proposed nuclear receptors binding motifs (DR2, DR4, IR1, and ER6) found in the reference sequence, only two motifs (DR2 and IR1) were turned in both observed genotypes of the studied cattle population. The reduction in the binding with nuclear receptors is usually attributed to the truncation existing in both genotypes because of the presence of the E115Ter nonsense mutation. The notion was easily made as both deleted motifs were positioned downstream of the observed E115Ter nonsense mutation.

Regarding the prediction of the intronic SNP effect on splicing events, it was found that no differences were observed in the splicing pattern after inserting the intronic SNP in the reference sequences to represent the variations acquired.

The consequences of E108D on leptin structure and function were studied extensively, in which the only observed nsSNP was found in the AA genotype (G94C). The consequences of this nsSNP were evaluated using different publicly available computational algorithms, namely SIFT, PANTHER, I-Mutant 2, SNAP2, and PROVEAN bioinformatics tools. Comparing the prediction of these methods, the deleterious consequence of this nsSNP was confirmed in only three of the five utilized tools. Usually, the SIFT tool is utilized first, which predicts whether an amino acid substitution affects protein function among related genes and domains over evolutionary time (Ng and Henikoff, 2006). SIFT predicted that p.Glu108Asp nsSNP was found to be tolerated, which showed a tolerated reliability index score of 0.6, which could not affect the protein function in this gene. The SIFT predicting tolerated characterization of this observed

nsSNP was further validated by PANTHER through investigation into the effect of this nsSNP on leptin function using HMM-based tool (Tang and Thomas, 2016). The latter tool did not agree with the SIFT prediction tool. The analysis of PANTHER revealed that p.Glu108Asp nsSNP reflected a probably damaging preservation time score (time > 450 my), thus PANTHER classified them as possibly damaging. However, the prediction of the functional impact of p.Glu108Asp nsSNP on the biological function of leptin was further investigated using the PROVEAN tool (Choi et al., 2012). In contrast to the PANTHER tool, PROVEAN revealed that this missense mutation was not deleterious (Table 5). It predicted that p.Glu108Asp was neutral (below -2.5). To add another layer of confirmation, the effect of this nsSNP was also analyzed using I-Mutant 2.0. It gave the result in the form of the effect of mutants on the stability of protein with a reliability index at pH 7.0 and temperature 25°C (Capriotti et al., 2005). The p.Glu108Asp nsSNP showed a decrease in the stability of leptin protein. SNAP2 server was also utilized in this regard (Smigielski et al., 2000), and it also predicted that this snSNP had a slight damaging effect (score > 0).

Table 4 Possible changes in reference, AA and AB genotypes of both A) intron 1 SNP and B) exon 3 SNPs with their NHR binding sites as analyzed by the NHR-scan program.

	A) Intron 1							
	Site type	**Sequence**	**Start**	**End**	**log (Viterbi probability)**	**log (Forward probability)**	**log (background state probability)**	**Forward background**
Ref., AB, AA								
	.....	.....	.....	...	.....	.....	.....	.....
	B) Exon 3							
	Site type	**Sequence**	**Start**	**End**	**log (Viterbi probability)**	**log (Forward probability)**	**log (background state probability)**	**Forward background**
	DR2	TGACCTGGAGAACC	4	17	-21.5153	-20.8028	-22.3315	1.5287
Ref.	DR4	GGGTCACTACAGGACA	161	176	-22.1397	-22.0801	-25.1242	3.0441
	IR1	AGGTCAGGGCCCT	69	81	-19.7017	-19.2096	-20.9351	1.7255
	ER6	TGAGCTTCTGGCTGTCCA	253	270	-27.8068	-27.1595	-27.9168	0.7573
AA and AB	DR2	TGACCTGGAGAACC	4	17	-21.5153	-20.8028	-22.3315	1.5287
	IR1	AGGTCAGGGCCCT	69	81	-19.7017	-19.2096	-20.9351	1.7255

Table 5 List of possible effects and consequences of the observed g. 93264127G>C nsSNP on exon 3 of leptin structure and function as determined by five bioinformatics tools

Amino acid change	**SIFT**	**PANTHER**	**PROVEAN**	**I-Mutant 2.0**	**SNAP2**	
p.E108D	0.6^{RI*}	220^{PT*}	-0.552^{CF*}	7^{RI*}	1^{*EC}	**Score**
	Tolerated	Possibly damaging	Neutral	Decrease	Effect	**Prediction**

^{RI*}, reliability index score; ^{PT*}, preservation time; ^{CF*}, cut-off = -2.5; ^{*EC}, expected accuracy = 53

The 3D structure of the native leptin model generated through RaptorX was visualized using PyMol. The leptin native blood circulating protein had 146 amino acid residues. RaptorX predicted that the structure had two highlighted SNPs; the first one is positioned at 108, in which the amino acid Glu (E) (Fig. 3A) was changed to the amino acid Asp (D) (Fig. 3B) while the second highlighted SNP was a nonsense mutation, in which the leptin was truncated at position 115 in both observed genotypes (Figs. 3B and C). All of these 146 (100%) residues were modeled and only 14 (9%) positions were predicted as disordered, while the truncated leptin had 37 (32%) positions predicted as disordered. Secondary structures of the native leptin model revealed 64% helix, 1% beta sheet, and 34% loop structures, while the secondary structure of the truncated *LEP* protein model revealed 53% helix, 2% beta sheet and 43% loop structures (Table 6). The solvent accessibility was divided into three states by 2 cut-off values, 10% and 42%. A value less than 10% was identified as buried, larger than 42% value was identified as exposed and between 10% and 42% was identified as a medium. Regarding solvent access, slight noticeable differences were found between the native and truncated leptin. The proportions of exposed, medium and buried regions in the native leptin were 23%, 56%, and 19%, respectively, while in the truncated leptin proteins were the same except the AB leptin genotype had 24% of exposed regions and the AA leptin genotype had 57% of the medium region. Overall, uGDT (GDT) values were 115 (79) and 88 (77) for the native and truncated leptin genotypes. uGDT is the unnormalized GDT (global distance test) score. For a protein with > 100 residues, uGDT > 50 is a good indicator. For a protein with < 100 residues, GDT > 50 is a good indicator. If a model has acceptable uGDT (> 50) but lower GDT (< 50), it indicates that only a small portion of the model may be good (Patel et al., 2015). *P*-value is the likelihood of a predicted model being worse than the best of a set of randomly-generated models for this protein (or domain), so *p*-value evaluates the relative quality of a model. The smaller the *p*-value, the higher the quality of the model. For alpha proteins, a *p*-value less than 10^{-3} is a good indicator. For mainly beta proteins, a *p*-value less than 10^{-4} is a good indicator. For this model of leptin, RaptorX predicted *p*-values of 2.47e-05 for the native protein and 4.61e-05 for the truncated leptin. The nature of the polar interaction of the observed E108D was tested by PyMol to unravel its possible role in the

conversion of the nature of the native amino acid binding with its surrounding residues in leptin. It was found that E108 in the native protein had two polar interactions with Gly111 of 3.8 Å and 5.5 Å lengths (Fig. 4A), while the altered amino acid D108 formed two polar interactions, but with two residues; the first one with Gly111 of 3.1 Å and the second with Ser106 of 3.2 Å (Fig. 4B).

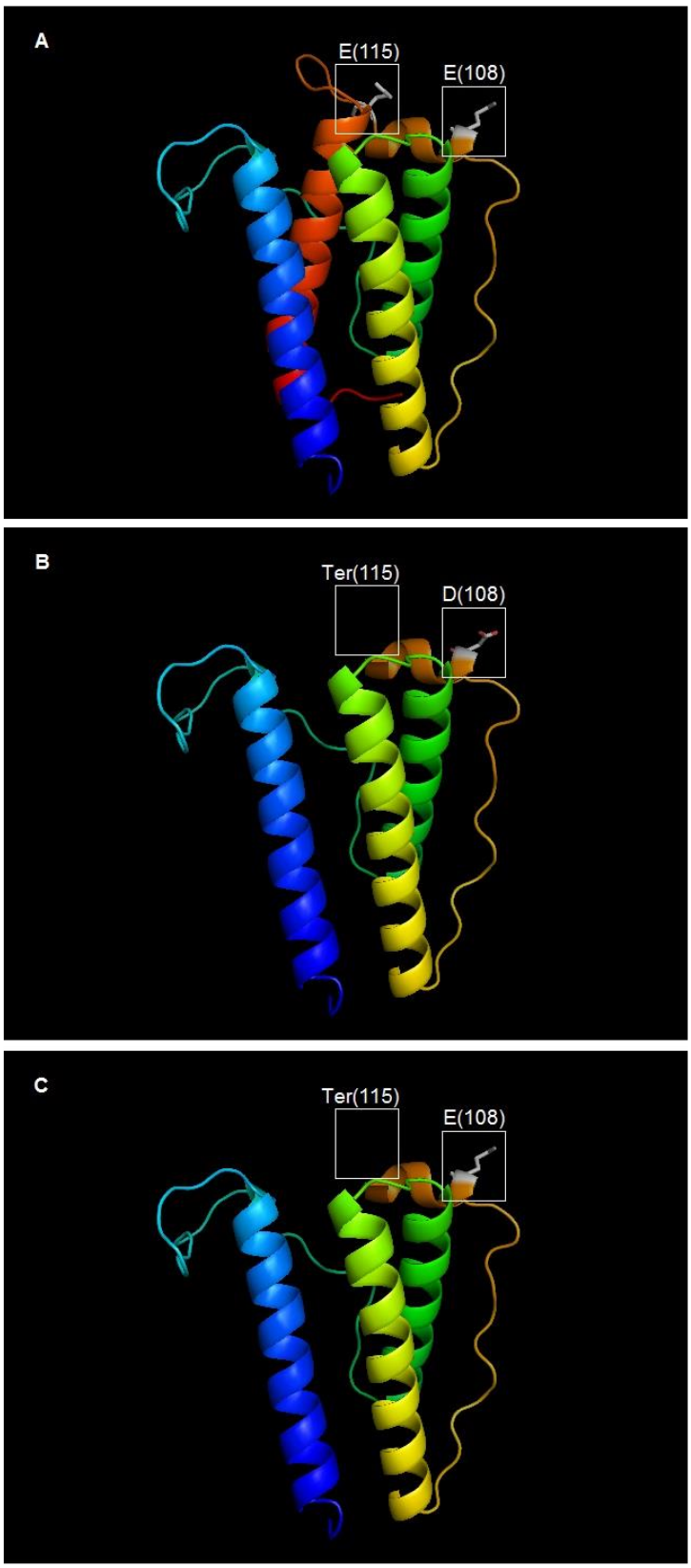

Figure 3 Postulated three-dimensional figures of the blood circulating bovine leptin showing the change of two amino acids, "E(108) and E(115) of the reference protein (A) into amino acids D(108) and Ter(115) of the AA genotype (B), and to only Ter(115) of the AB genotype (C) of Holstein cattle.

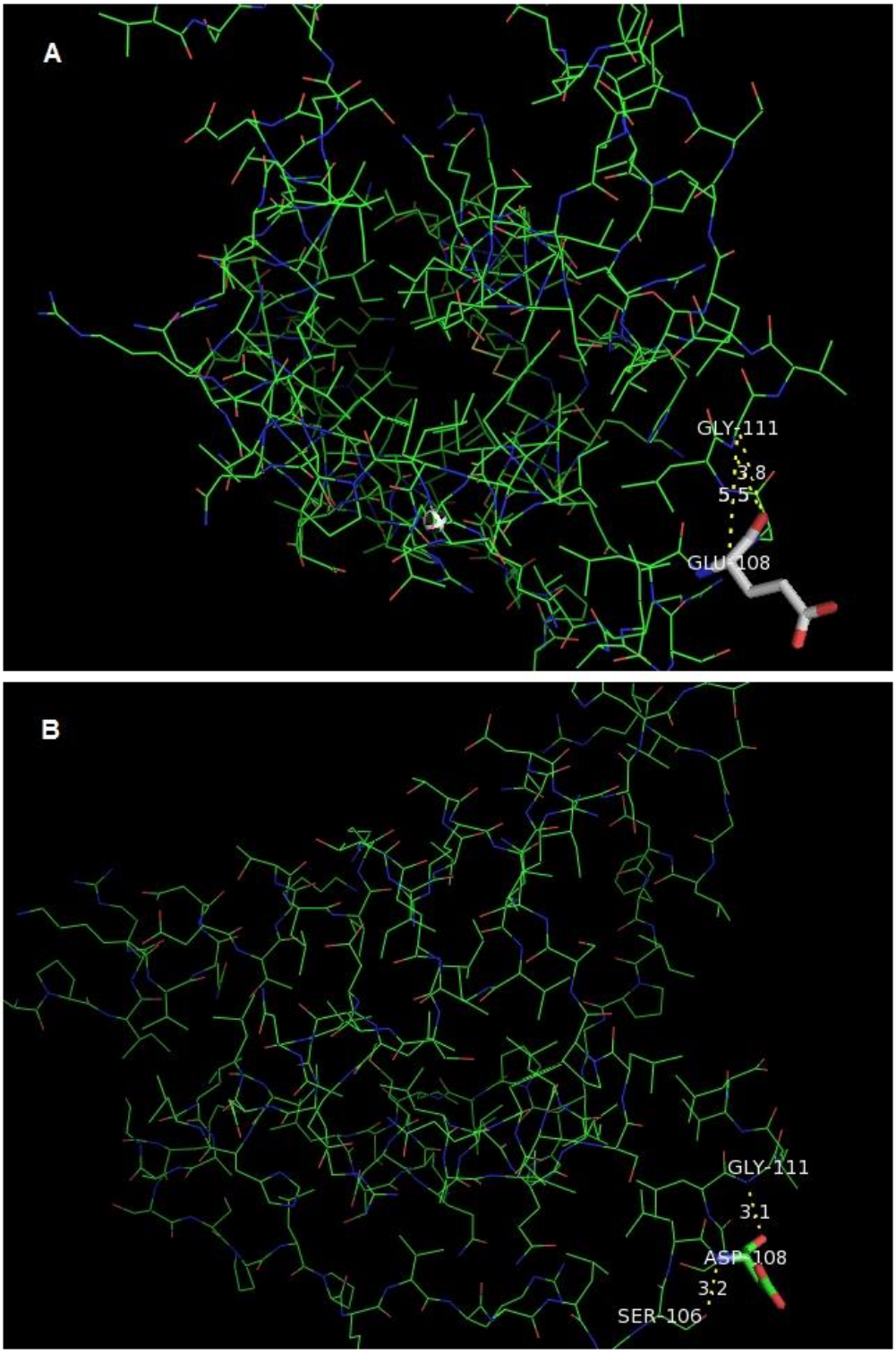

Figure 4 The polar interaction pattern of the native Glu (E) residue with vicinal residue (**A**), and the interaction pattern of altered Asp (D) residue with vicinal residue (**B**) in the Holstein leptin. The connection (yellow dotted lines) lengths between residues were measured in Å dimensions.

Table 6 RaptorX three-dimensional structure prediction parameters for native leptin as well as truncated leptin of both AB and AA genotypes

Parameter	Native leptin	Truncated leptin (AB genotype)	Truncated leptin (AA genotype)
p-value	2.47e-05	4.54e-05	4.61e-05
Overall uGDT (GDT)	115 (79)	88 (77)	88 (77)
Modeled residues	146 (100%)	114 (100%)	114 (100%)
Predicted disordered positions	14 (9%)	37 (32%)	37 (32%)
Secondary structures	64%H, 1%E, 34%C	53%H, 2%E, 43%C	53%H, 2%E, 43%C
Solvent access	23%E, 56%M, 19%B	24%E, 56%M, 19%B	23%E, 57%M, 19%B

Phylogenetic tree of truncated Leptin genotypes: A phylogenetic tree was made by using MEGA5, which is user-friendly software for mining online databases. MEGA5 is a collection of maximum likelihood (ML) analyses for inferring evolutionary trees, selecting best-fit amino acids-based substitution models, inferring ancestral states and sequences (along with probabilities), and estimating evolutionary rates site-by-site (Tamura et al., 2011). The phylogenetic tree constructed for our observed exon 3 AA and AB genotypes detected distinctive features among them with respect to each other. Although 113 amino acids were shared between the two genotypes, one difference was presented by E108D, which noticeably changed the localization of genotype AA in the tree. This new amino acid substitution made the AA genotype occupy a singular category in the tree. However, the thirty-one amino acid truncation made the two genotypes so distinct in terms of position and evolutionary relationships (Fig. 5).

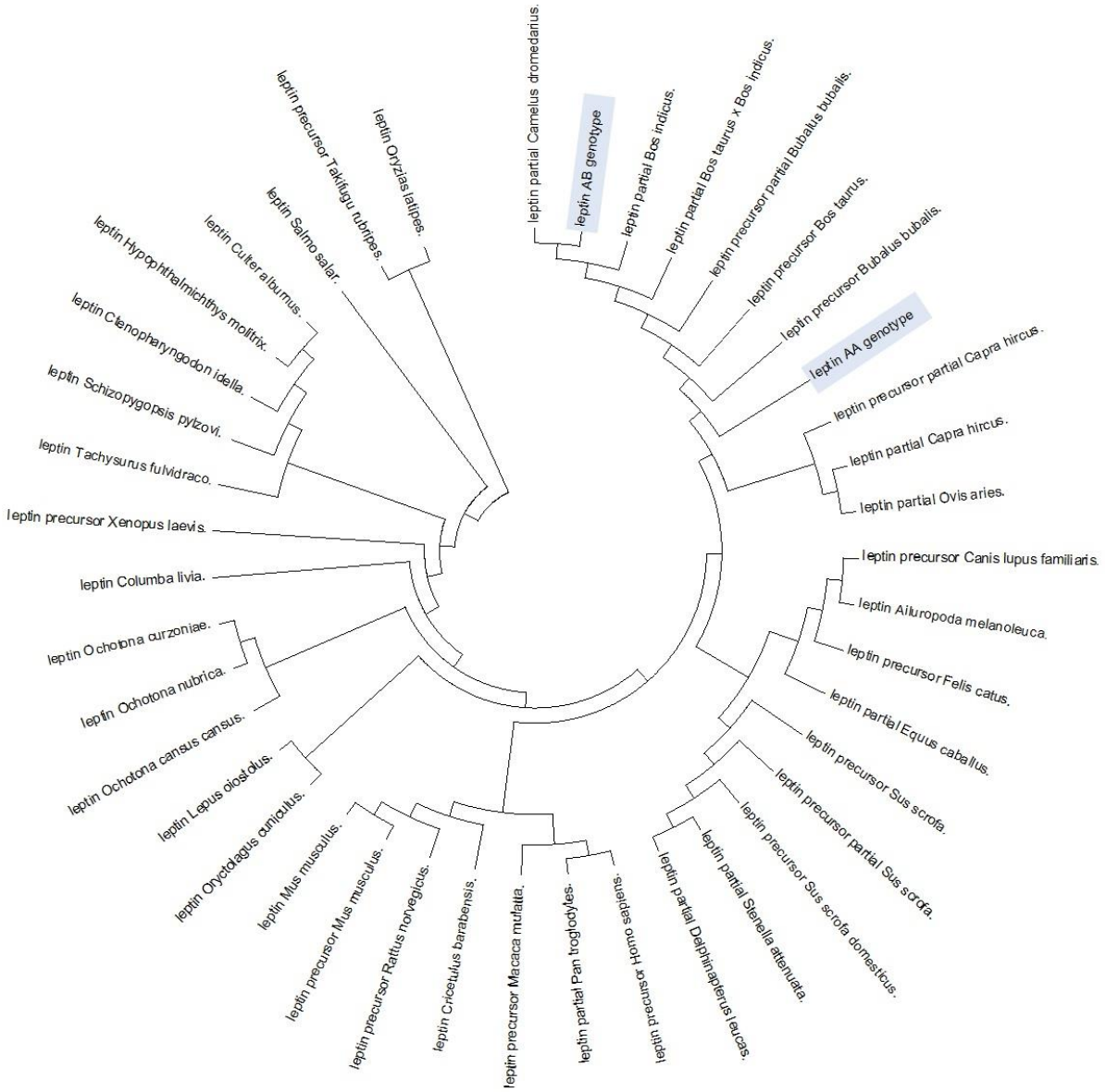

Figure 5 A phylogenetic tree of bovine (*Bos taurus*) native leptin and its observed highlighted AA and AB genotypes in comparison with other leptin sources

Discussion

Since the formation of truncated proteins does not occur often *in vivo*, the identification of naturally occurring deleterious gene mutations may provide an insight into the functionally important regions of several proteins. In this study, a novel nonsense mutation in the bovine leptin was described. The polymorphism of 117 amino acids (70%) out of 167 amino acids of the whole *LEP* protein, including 48 amino acids of all exon 2 region (about 29%) and 69 amino acids of a part of exon 3 (about 41%), was covered in this study. Two types of genetic data were performed, which included the *in vitro*-based *LEP* gene PCR-SSCP sequencing, and several *in silico*-based computational tools that were utilized to analyze the final consequences of these observed SNPs on both

leptin structure and function in the highly important milk producing Holstein population.

Four SNPs were indicated in this study, the first three were an intronic SNP, an exonic sSNP, and an exonic nsSNP; it was found through several computational tools that the effect of these SNPs ranged from non-effective to mildly effective on protein structure and function. Instead, the most important observation of this study lies behind the fourth SNP, the nonsense SNP, which undoubtedly should be attained a particular attention because of the novelty of the deleterious consequences of this SNP on leptin structure and function. Nonetheless, the first three observed SNPs were analyzed computationally. Concerning the intronic SNP, no effect of this SNP on the leptin splicing pattern was found as it was predicted by the ESEfinder tool (Cartegni et al., 2003), while the exonic sSNP has been given a non-effective role as it was deduced from the NHR-scan. Conversely, the situation of the third SNP was different, since it was a nsSNP. It is known that the understanding of the functions of some of the nsSNPs can greatly help in understanding the bovine genotype/phenotype variations which may have several effects on some milk synthesis characterization. The nsSNPs may affect gene expression by modifying DNA and transcription factor binding, inactivate active sites of enzymes or change splice sites, thereby producing defective gene products. Therefore, the observed p.Glu108Asp in the genotype AA was also given a special attention in this study. To determine the functional effect of this nsSNP in leptin, several widely used *in silico* tools were employed in this study. However, these tools gave different predictions on the deleterious effect of p.Glu108Asp nsSNP on leptin. The difference in the results of these prediction tools is due to the differences in the features utilized by the methods, therefore dissimilarities of the outcomes were expected to occur at some points (de Alencar and Lopes, 2010). In addition to the previously mentioned bioinformatics tools, the polar interaction of this nsSNP with other residues was studied, in which, a slight modification to the binding pattern was noticed and measured. Although relatively discrepant results were noticed, the overall prediction of the several bioinformatics tools indicates that this nsSNP may have a moderate effect on both leptin protein and structure. Conversely, the observed E115Ter nonsense mutation causes deletion of thirty-one amino acids and consequent premature termination of transcription to produce an incomplete leptin and potentially nonfunctional amino acid residues. The deleterious effect of such nonsense mutation and perhaps almost all stop mutations are unquestionable. However, the thing that led us to try to analyze the sort or the degree of such damage is the critical role of this protein in various crucial activities in the body. As a part of the highly sophisticated machinery involved in several metabolic pathways, leptin is not only an endocrine hormone in the brain and in different peripheral tissues in which leptin receptors are expressed, but it also acts as autocrine, and even paracrine hormone within tissues in several mammals, such as ruminants (Agarwal et al., 2009). Hence, the NHR-scan program was utilized to explore the possible role of the observed SNPs of this study in the change in the leptin characterizations with respect to its nuclear surface motifs (Sandelin and Wasserman, 2005). Interestingly, a noticeable reduction in the binding with the nuclear receptors was observed after deleting 177 nucleotides from the *LEP* reference sequences as observed with both AA and AB genotype nonsense mutation. This, in turn, suggests a possibly prohibitive role of this nonsense mutation in demolishing the leptin binding motifs, which may consequently affect the nuclear receptor-mediated metabolic pathway in which leptin is involved. Although the precise mechanism by which leptin is potentially interacting with nuclear receptors is unknown, this finding may pinpoint some initial data which may help with the precise understanding of these motifs which may change the substrates with which they are interacting. The NHR-scan program showed that both observed intronic SNP and exonic sSNP had no role in this alteration as all the binding changeability was noticed only in the deleted segment caused by the nonsense mutation.

Although *LEP* exons have a high mutation frequency, the presence of a nonsense mutation in a position that abolishes the coding of thirty-one amino acids of α-helix D was not expected in our study. Three receptor binding sites on mammalian four-helix leptin structure, which are important to leptin function, were identified. The leptin receptor binding site I is located in the C-terminus of helix D. It is a 50 amino acid long chain, which is positioned within 95-145 residues of mature blood circulating leptin. However, the exact function of helix D is unknown, but it exhibits unique structure which might allow leptin to specifically bind to its receptor through enhancing the activity of N-terminal through the Cys96-Cys146 disulfide bond. The mutation of leptin causing the deletion of one of the two cysteine residues leads the disappearance of the disulfide bond and twisted helix. This deletion sometimes renders the protein biologically inactive (Zhang et al., 1994). The latter notion is an inevitable outcome for the deletion of Cyst146 residue that accompanied with our Glu115Ter observed mutation. Nevertheless, other studies have shown that leptin is able to bind to its receptor and maintain energy homeostasis without the disulfide bond and kinked D helix (Imagawa et al., 1998). However, the mutations in the binding site I did not affect receptor binding but showed a modest effect on signaling (Peelman et al., 2004). Indeed, the Glu115Ter causes a misfolding in N-terminal, since the latter interconnects with the C-terminal in which the Glu115Ter occurs. Thus, it would not allow the N-terminal to maintain the correct conformation needed for binding. This idea is further supported by studies that show that the deletion of the C-terminal yields a less stable protein. Regarding leptin receptor binding site II, which is represented by the surface of helices A and D, this fragment is positioned within 85-119 residues of mature blood circulating leptin. It was found that leptin bound to its receptor at the interface of α-helices A and C (Hiroike et al., 2000). Mutations in the binding site II at the surface of helices A and C impaired receptor binding, but had only a limited effect on signaling (Peelman et al., 2004). Thus, this Glu115Ter may, in turn, reduces receptor binding. Moreover, it was found that the

administration of synthetic leptin peptides identified positions 85-119 as critical for appetite suppression and weight loss in obese mice, since the evolutionary, structural, and biochemical information implicated segments 85-119 as of special functional significance (Gaucher et al., 2003). On the other hand, the leptin receptor binding site III, which is not affected by this Glu115Ter nonsense mutation, is located at the N-terminus of helix D and extended from 1 residue into 94 residues of mature blood circulating leptin. It acts on direct binding with leptin receptor. However, it was folded within three turns and once it was interwoven with the C-terminal loop structure. Thus, no proper folding of N-terminus was performed in this study as the C terminus was completely deleted. However, the deletion of the N-terminal showed no effect on food intake and weight gain while the deletion of the C-terminal did, suggesting that the N-terminal is responsible for the binding of leptin to its receptor. Consequently, if leptin cannot bind to its respective receptor because of this observed nonsense mutation, the body is not able to regulate its energy expenditure. Indeed, the position at which this mutation occurs is somewhat confusing because of the intervention between C and D helices through the distorted E helix. Thus, besides being included within the E helix, the truncation of the Glu115Ter causes deletion of the downstream D helix. Another layer of complication is added when it was noticed in the present study that the helix D kept the integrity of helix C through the deleted Cys146. Therefore, this nonsense mutation may damage helix C in addition to the damage of both D and E helices. However, the presence of a nonsense mutation in leptin is not a common result in any cattle population. The presence of such mutation might be attributed to the nature of polymorphisms that were highly dependable on the type and location of the breed used. The results of this study may suggest high polymorphism gain as a result of the genetic adaptation to the extreme warm climates these cattle are exposed to (Berman, 2011).

Undoubtedly, the potential relationship of this stop mutation could be clearly hypothesized when it was accompanied by full phenotypic records for Holstein milk production traits. Nonetheless, this study has provided pilot data that filter out the likelihood of this damaging mutation to affect leptin function, which likely has an impact on the quality of some bovine milk components as deleterious nonsense mutation have a highly damaging influence on protein structure and interaction. However, a nonsense mutation in leptin was discovered in mice at position 105, in which Arg codon is converted into a termination codon (Hamann and Matthaei, 1996). Ultimately, a nonfunctional protein is produced and the idea of the production of a functional truncated leptin is not easy to accept or at least is not easy to prove. Eventually, nonsense mutations frequently cause severe deterioration, since the premature termination of translation creates truncated proteins that are usually unstable. This discrepancy is a remarkable finding and indicates that truncation or even the absence of the last thirty-one amino acid fragments is not completely deleterious on bovine milk production. If a considerable deleterious effect of this mutation on milk production is assumed, the abolishing effect of this mutation could not be assumed as long as this population is currently utilized effectively in milk production in the local market.

Regarding milk trait, if the common idea that supports the loss of leptin activity after being prematurely terminated is accepted, it should be concluded that this loss of activity does not correlate with a complete loss of milk production.

In conclusion, this study provides an indication for the presence of a nonsense mutation in bovine leptin that may have significant consequences on both bovine milk and weight traits. An association study is mandatory in this regard to give an adequate insight into this phenomenon.

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บทคัดย่อ

การแปรผันของลำดับดีเอ็นเอชนิดหนึ่ง (สนิป, SNP) ของยีนเลปติน ในโคนมโฮลสไตน์

โมฮัมหมัด บากู เอส แอล-ชูฮา[1*] ซาร่า เอ็ม เอ แอล-ลามี่[1] ฮาเฟด เอ็ม เอ แอล-ทาย[2]
ทัทเรีย เอ็ม แอล-ทูน่ไวนี[1] อาดิล เฮช ราดี[1]

เลปตินเป็นฮอร์โมนที่มีความสำคัญในการควบคุมเมตาโบลิสมในร่างกาย เช่น ควบคุมน้ำหนัก ควบคุมการกินอาหาร การใช้พลังงาน ระบบภูมิคุ้มกันและระบบสืบพันธุ์ การศึกษานี้มีวัตถุประสงค์ในการตรวจหา การแปรผันของลำดับดีเอ็นเอชนิดหนึ่งของยีน Bovine leptin (LEP) โครงสร้างและหน้าที่ของยีนดังกล่าวในโคนมโฮลสไตน์ ทำการเจาะเก็บเลือดจากโคนม จำนวน 60 ตัว นำมาสกัดดีเอ็นเอ และใช้ไพรเมอร์ที่จำเพาะจำนวน 2 คู่ ทำการทดลองด้วยเทคนิค PCR และ SSCP รูปแบบปรากฏทั้งสองของ SSCP ซึ่งแสดงถึง 2 จีโนไทป์ (AA, AB) ถูกตรวจพบใน 2 อัลลิล SNPs จำนวน 4 รูปแบบ ในการศึกษาครั้งนี้ คือ Intronic, exonic synonymous, exonic non-synonymous, and nonsense SNP SNP รูปแบบใหม่ที่ค้นพบและมีความน่าสนใจคือ p.E115Ter nonsense mutation ซึ่งเกิดขึ้นที่ส่วน premature termination และอยู่ที่ส่วนท้ายกรดอะมิโนที่ 31 ส่วนของลำดับเบสที่หายไปคือ α-helix E, α-helix D, and Cyst146 ซึ่งแสดงถึงการผ่าเหล่าของยีนที่เกิดขึ้น ซึ่งส่งผลต่อการปรากฏของตัวรับของยีนเลปติน ซึ่งมีรายงานการผ่าเหล่าของยีนนี้ มีผลต่อผลผลิตน้ำนมในกลุ่มประชากรโคนม อย่างไรก็ตามในการศึกษาครั้งนี้ ไม่ได้แสดงผลของความสัมพันธ์ระหว่างการผ่าเหล่าของยีนต่อผลผลิตของโคนม

คำสำคัญ: โคนมโฮลสไตน์ ยีนเลปติน น้ำนม nonsense mutation sequencing SNPs

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