LEPTIN GENE SNPS AND THEIR EFFECT ON MILK TRAITS IN EGYPTIAN BUFFALO (*BUBALUS BUBALIS*)

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ABSTRACT

Leptin plays an important role in the regulation of development of the mammary gland and its polymorphisms were shown to be associated with milk quantity and quality in cattle. However, no SNP was detected and no association studies was conducted on leptin gene in Egyptian buffalo. Therefore, the objective of this study was to detect leptin gene polymorphisms and to determine associations between these polymorphisms and milk traits in Egyptian water buffalo. A PCR product of 400bp, including intron 2 of leptin gene was amplified using PCR and subsequently, subjected to sequence analysis to identify its different allelic patterns. Two novel SNPs,G247T at nucleotide number 963 and T282G at nucleotide number 998, were detected in LEP intron 2 in Egyptian buffaloes. The associations analysis revealed no significance association between these SNPs and milk traits(P>0.05) in all examined animals. Consequently, our results indicated that the LEPG247T and T282GSNPs does not influence milk yield or milk composition in Egyptian buffalo and these SNPs could not be used in a marker assisted selection program to improve milk traits in Egyptian buffalo.

Keywords: Leptin, Egyptian buffalo, PCR, sequencing, SNPs, milk traits.

INTRODUCTION

The water buffalo represents an important dairy animal in Egypt. The unique ability of buffalo to survive under highly difficult conditions of nutrition and management, long productive life, and the ability to change poor quality rich in complex carbohydrate plants to high quality milk given it a competitive edge to other milk producing animals and suitability to the lives of small farmers ***(El-Magd et al., 2013; Marai et al., 2009)***. Egyptian buffalo milk has higher nutritive value as it contains higher levels of total solids, crude protein, fat, calcium, phosphorus and lower level of cholesterol (total and free) as compared to those of native and imported dairy breeds ***(Ahmad et al., 2013; El-Magd et al., 2013; Enb et al., 2009)***.

Although there are many advantages to raise water buffalo as described above, these animals remain underutilized and their production does not cover the local market requirement. Phenotypic selection is not a successful breeding method for traits such as milk production, due to the low heritabilities for these traits ***(Hansen, 2000)***. The integration of molecular data into breeding program in addition to phenotypes helps to the increase genetic gain and in consequence in increase animal productivity. Genotype based selection depends on identification of molecular markers in the genes affecting economic traits ***(Dekkers, 2004)***. Molecular markers, revealing polymorphisms at the DNA level, are now key players in animal genetics, one of the excellent marker is single nucleotide polymorphism (SNP) ***(Vignal et al., 2002)***.

A lot of candidate genes have been found to influence milk yield, fat and protein content and protein quality. These genes either share direct in biochemical pathways for the synthesis of milk components, as diacylglycerolacyl transferase 1 (DGAT1) ***(Smith et al., 2000)***, and α-

Lacalbumine (α-LA) ***(Miglior et al., 2007)*** or affect mammary gland development and differentiation which in turn affect milk yield as Leptin (*LEP*) gene ***(Neville et al., 2002)***.Leptin gene consists of 3 exons and 2 introns ***(delaBrousse et al., 1996; He et al., 1995)*** and has been mapped to chromosome 4 in bovine ***(Stone et al., 1996)***. Although adipose tissue is the primary source of leptin, it is also expressed in a mammary tissue ***(Aoki et al., 1999; Smith-Kirwin et al., 1998)***, specifically in the alveolar epithelial cells of the mammary gland ***(Sayed-Ahmed et al., 2003)***. Leptin plays an important role in the regulation of development of the mammary gland. It influences the branches of ducts and the proliferation of the mammary epithelial cells as well as the expression of milk protein genes ***(Lin and Li, 2005).*** Therefore, some studies have been reported associations between leptin gene polymorphisms and carcass merit ***(Buchanan et al., 2002)***, and milk quantity and quality ***(Buchanan et al., 2003; Liefers et al., 2003)*** in cattle.

Little efforts have been made to improve Egyptian buffalo genetic potentiality for milk production. Therefore, the aim of this study is to identify *LEP* SNPs and to study their association with milk yield and milk quality traits in Egyptian water buffalo.

MATERRIALS AND METHODS

Sampling and DNA extraction:

This study involved 200 pure Egyptian water buffalo (*Bubalus bubalis*) kept on a farm located in Nucleus Herd, Nataff Gedeed station of Mahalet Mousa farm, Kafrelsheikh governorate.All records of milk production traits in Beheiry buffaloes were collected from farm records to be used for statistical analysis. Records covered the period from 2010 till 2013.

Blood samples were collected by jugular vein puncture into vacutainer tubes containing an anticoagulant (disodium EDTA) and kept in ice box. The genomic DNA was extracted using Gene JET genomic DNA extraction kit following the manufacturer protocol ***(Fermentas, #K0721/USA).***

Polymerase chain reaction:

A partial sequence of leptin geneintron 2 was amplified by PCR using primers (Table 1) designed by Primer 3.0 software based on the published sequences of Indian buffalo (GenBank accession number, AY495587).

Table (1): Primer sequences, annealing temperature (Ta) and PCR product characteristics of intron 2 of LEP gene

Gene	Forward primer ('5-'3)	Reverse primer ('5 - '3)	Ta (°c)	Size (bp)	Localization
LEP	CCCAACAAGATCTTC CCAACCCAGG	TGTCCCCGCTTCTGGC TACCT	52	399	Intron 2

The PCR was carried out in a reaction volume of 50 µL, containing 4.0 µL DNA template (approximately 100 ng), 1.0 µL (0.20 mM) dNTP, 5.0 µL buffer, 3.0 µL (2.5 mM) MgCl2, 2.0 µL 10µmol/L forward primer, 2.0µL 10µmol/L reverse primer, 1.0 µL 10X Taq DNA polymerase (5 U/µL, Fermentas, #K1071, European Union), and 32µLnuclease free water. Thermal cycling parameters were as follows: initial denaturation at 94°C for 5 min, 35 cycles of amplification (94°C for 40 s for DNA denaturation, annealing 52°C for 1 min, extension at 72°C for 1 min) and final extension at 72°C for 10 min. The samples were held at 4°C. PCR products were resolved by electrophoresis on 2% agarose gel in 1X TBE, stained with ethidium bromide and visualized with UV light of gel documentation system (Biometra Biomedizinische Analytik, GmbH).

DNA sequencing:

PCR products with expected size were purified using PCR purification kit following the manufacturer protocol (Jena Bioscience # pp-201×s) to remove primer dimmers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities ***(El-Magd et al., 2013)***. The purified PCR products were sequenced in automated sequencer (Applied Biosystem, USA). The Sequences were analyzed using the Chromas Lite 2.1 program (http://technelysium. com. au/? page_id=13) and the identity of the sequenced PCR product was examined using Blast search against GenBank database of Indian buffalo (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments, annotations and assembly of the sequences were performed using Geneious 4.8.4 software http://www.geneious.com/web/geneious/home.

Statistical analysis:

The effect of different genotypes on various milk production traits were analyzed by least squares method using a mixed model with fixed effect of genotype.

- $Y_{ijk} = \mu + G_i + M_j + B_k + e_{ijk}$
- Y_{ijk} is the analysed trait of the cow,
- μ is the overall population mean,
- G_i is the fixed effect of the ith genotype (1,2),
- M_j is the fixed effect of jth season of calving (1,2,3),
- B_k is the fixed effect of kth year of calving (1, 2),
- e_{ijk} is the random error.

RESULTS

A 399 bpfragment of LEP intron 2 was amplified using PCR (Fig. 1).

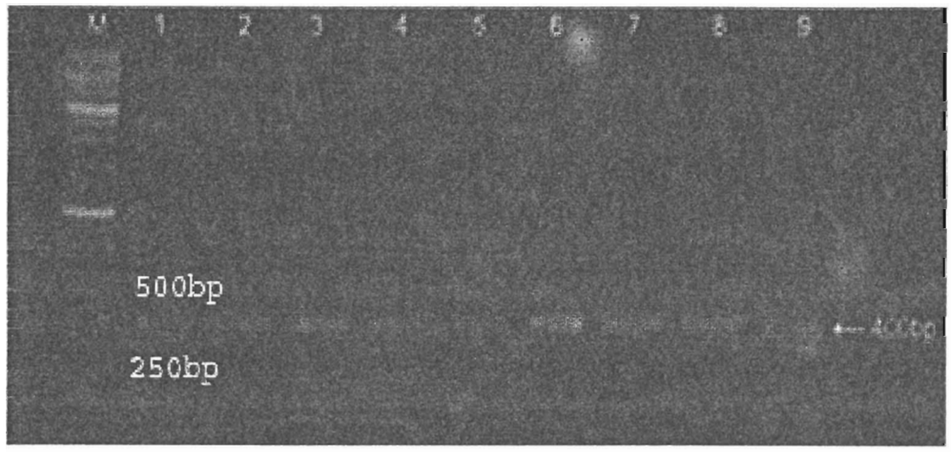

Fig. (1): Ethiduim bromide stained agarose gel showing amplified 400bp PCR products of LEP gene from nine animals (lanes 1-9). M represents a 1Kb ladder.

After purification of these fragments, sequencing was carried out in order to verify the identity of the PCR product and to detect any SNPs. The obtained sequences were examined against previously known sequences on Indian buffalo published in GenBankdatabase using Blast search. The results of this step confirmed that the sequences, as expected, showed high similarity with Indian buffalo *LEP*.

We have detected two novel SNPs; G247T at nucleotide number 963 (Fig.2) and T282G at nucleotide number 998(Fig.3), in intron2 of *LEP* gene among all examined Egyptian buffaloes.

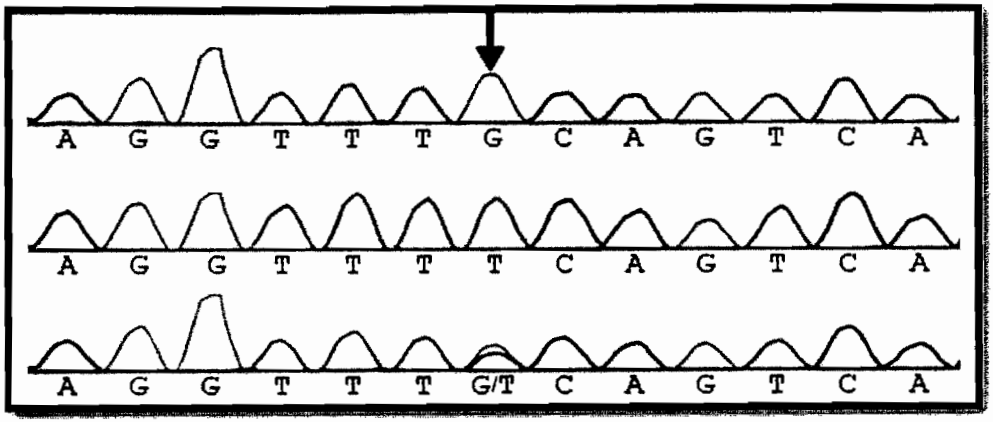

Fig. (2): Nucleotide sequences of LEP intron 2 show a G247T SNP at nucleotide number 963. The arrow indicates the position of the SNP.

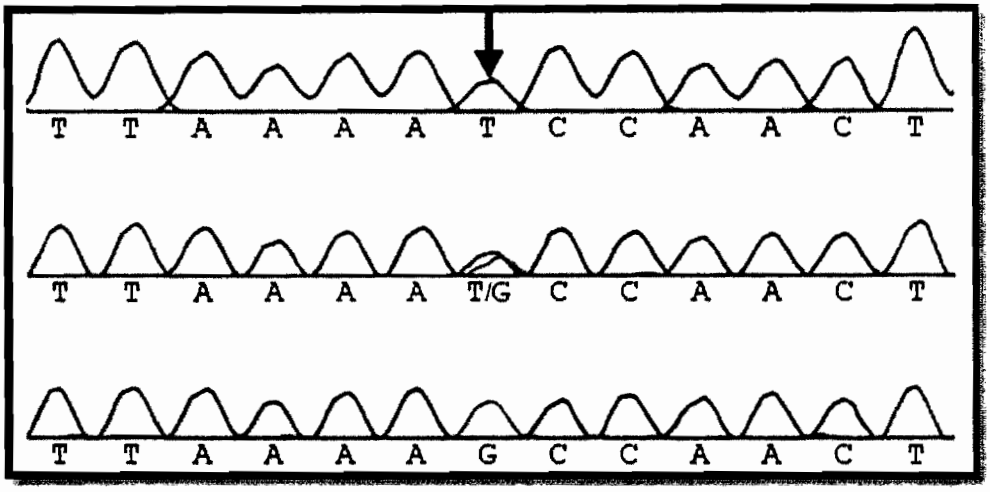

Fig. (3): Nucleotide sequences of LEP intron 2 show a T282G SNP at nucleotide number 998. The arrow indicates the position of the SNP.

Least squares mean (LSM) for milk traits related to different sequence variants obtained by statistical analysis were given in Table 2. We did not detect significant association betweenG247T and T282G SNPs in LEP intron 2 withany of studied milk traits($P>0.05$).

Table (2): Least squares mean (±SE) of different production traits for G247T and T282G SNPs of *LEP* intron 2. Lactation length is given in days and milk, fat, protein, lactose and total solid yield in Kg.

Traits	GG/TT	TT/TT	GT/TG	GT/GG
Lactation length	261.54±6.62	260.43±6.46	262.47±6.52	260.96±7.24
Milk yield	2052.57±70.53	2059.64±51.35	2054.56±64.59	2056.72±60.92
Fat percentage	6.40±0.03	6.35±0.03	6.38±0.04	6.29±0.04
Protein percentage	4.10±0.04	4.14±0.02	4.16±0.03	4.13±0.03
Lactose percentage	5.36±03	5.32±03	5.29±03	5.33±03
Solid percentage	17.02±0.41	17.10±0.33	17.24±0.38	17.30±0.43

DISCUSSION

Milk production trait is considered as one of the most economically valuable traits in animal production. Therefore, breeding for more and high quantity and quality milk is a main goal in animal breeding programs. Milk traits are polygenic and quantitative in nature, controlled by a lot of genes. *LEP* gene is a lactogenic gene, which is considered to be a positional candidate gene for milk traits in bovine ***(Liefers et al., 2003)***. Several studies have been conducted to identify the relationship between leptin gene polymorphism with traits of economic importance in cattle as, carcasse traits ***(Schenkel et al., 2005)***, feed intake ***(Nkrumah et al., 2004.)***, milk traits ***(Buchanan et al., 2003).***

To our knowledge, this is the first association study applied on LEP gene of Egyptian buffalo. In this study, two SNPs a G247T at nucleotide number 963 and T282G at nucleotide number 998 in intron 2 of LEP gene were identified in Egyptian buffalo. In consistence, some novel SNPs were detected in buffalo but their association analysis has not studied yet. Twelve new SNPs were detected in buffalo leptin useful for association studies, C1221T in exon 2, in exon 3 G3195A (resulting in a Arg to Gln substitution in the codon 159), G3318A and G3434A while SNPs T1015C, C1071T, G1072A, T1081C, T1131G,T1143C , T1145G and A1151G are all intronic ***(Orru et al., 2007)***.

Generally, the polymorphisms at splice junctions of exon/intron boundaries were considered as significant due to the insertion of extra nucleotides in mRNA thereby a change in amino acid sequence in proteins. In the present work, either G247T SNP or T282G was not at the splice junctions. However, some studies signified that the changes in non-coding DNA sequences often manifest themselves in clinical and circumstantial malfunctions ***(Mattick and Gagen, 2001)***. Numerous genes in these protein non-coding regions, encode micro RNAs, which are responsible for RNA-mediated gene silencing through RNA interference (RNAi)-like pathways. Intron-derived miRNA (Id-miRNA) is a new class of miRNA, derived from the processing of gene introns. The intronic miRNA requires type-II RNA polymerases (Pol- II) and spliceosomal components for their biogenesis. Several kinds of Id-miRNA have been identified in C. elegans, mouse, and human cells, however, neither function nor application have been reported. It was reported that intron-derived miRNAs are able to induce RNA interference in not only human and mouse cells, but also in zebrafish, chicken embryos, and adult mice, demonstrating the evolutionary preservation of intron-mediated gene silencing via functional miRNA in cell and in vivo ***(Lin et al., 2006)***. These reports suggested an intracellular miRNA-mediated gene regulatory system, fine-tuning the degradation of protein coding messenger RNAs. Along similar lines, the polymorphisms in intron 2 of *LEP* gene may be useful for future studies to know their role for the regulation of *LEP* mRNA function.

In this study, nosignificant association was observed between G247T SNP, T282G SNPs and milk production and quality traits in Egyptian buffaloes. In contrast, In promoter region, two SNPs in the bovine leptin promoter (A1457G and C963T) are associated with leptin concentrations during the periparturient period and with dairy traits

(Liefers et al., 2005). In exon 2 of leptin, A252T, which introduces an AA change from Tyr to Phe, was associated with higher milk, fat, and protein yields ***(Sadeghi et al., 2008)***. In addition, C73T SNP, which introduces an AA change from Arg to Cys R25C, was significantly associated with increased milk and protein yield without changing yield of milk fat ***(Buchanan et al., 2003)***. Cows homozygous for the C allele of C73T SNP were less productive (lower fat, true protein and lower milk yield) than those carrying the CT and TT genotypes ***(Chebel et al., 2008)***. The LEP haplotype CCGTTT (corresponding to leptin SNP C207T, C528T, A1457G, C963T, A252T, and C305T) significantly associated with milk yield, in cows ***(Banos et al., 2008)***. In exon 3 of leptin gene, a C177T SNP, introducing an AA change from Ala to Val, was associated with crow rump length, heart girth and milk production traits ***(Clempson et al., 2011)***.

In conclusion, G247T and T282G SNPs in the intron 2 of LEP gene does not influence milk yield or milk composition in Egyptian buffalo and these SNPs could not be used in a marker assisted selection program to improve milk traits in Egyptian buffalo. Considering the economic importance of the milk to the livestock industry, it appears clearly essential to further research on *LEP* in the buffalo.

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REFERENCES

- ***Ahmad, S., Anjum, F. M., Huma, N., Sameen, A., Zahoor, T., 2013.*** Composition and physico-chemical characteristics of Buffalo milk with particular emphasis on lipids, proteins,minerals, enzymess and vitamins. The Journal of Animal and Plant Sciences. 23(1 Suppl.), 62-74.
- ***Aoki, N., Kawamura, M., Matsuda, T., 1999.*** Lactation-dependent down regulation of leptin production in mouse mammary gland. Biochimica Et Biophysica Acta-General Subjects. 1427, 298-306.
- ***Banos, G., Woolliams, J. A., Woodward, B. W., Forbes, A. B., Coffey, M. P., 2008.*** Impact of single nucleotide polymorphisms in leptin, leptin receptor, growth hormone receptor, and diacylglycerol acyltransferase (DGAT1) gene loci on milk production, feed, and body energy traits of UK dairy cows. Journal of Dairy Science. 91, 3190-3200.
- ***Buchanan, F. C., Fitzsimmons, C. J., Van Kessel, A. G., Thue, T. D., Winkelman-Sim, D. C., Schmutz, S. M., 2002.*** Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels. Genetics, Selection, Evolution . 34, 105-116.
- ***Buchanan, F. C., Van Kessel, A. G., Waldner, C., Christensen, D. A., Laarveld, B., 2003.*** An association between leptin single nucleotide polymorphism and milk and protein yield . Journal of Dairy Science. 86, 3164– 3166.
- ***Clempson , A. M., Pollott , G. E., Brickell , J. S., Bourne , J. S., Munce , N., Wathes, D. C., 2011.*** Evidence that leptin genotype is associated with fertility, growth, and milk production in Holstein cows. Journal of Dairy Science 94, 3618–3628.
- ***Dekkers, J. C., 2004.*** Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. Journal of Animal Science . 82 E-Suppl, E313-328.

- ***DelaBrousse, F. C., Shan, B., Chen, J. L., 1996.*** Identification of the promoter of the mouse obese gene. Proceedings of the National Academy of Sciences of the United States of America. 93, 4096-4101.
- ***El-Magd, M. A., Abbas, H. E., El-kattawy, A. M., Mokhbatly, A., 2013.*** Novel polymorphisms of the IGF1R gene and their association with average daily gain in Egyptian buffalo (Bubalus bubalis). Domestic animal endocrinology. 45, 105-110.
- ***Enb, A., Abou Donia, M. A., Abd-Rabou, N. S., Abou-Arab, A. A. K., El-Senaity, M. H., 2009.*** Chemical Composition of Raw Milk and Heavy Metals Behavior During Processing of Milk Products. Global Veterinaria . 3, 268-275.
- ***Hansen, L. B., 2000.*** Consequences of selection for milk yield from a geneticist's viewpoint. Journal of Dairy Science . 83, 1145-1150.
- ***He, Y. F., Chen, H., Quon, M. J., Reitman, M., 1995.*** The Mouse Obese Gene - Genomic Organization, Promoter Activity, and Activation by Ccaat/Enhancer-Binding Protein-Alpha. Journal of Biological Chemistry. 270, 28887-28891.
- ***Liefers, s. c., Te Pas, m. F. W., Veerkamp, R. F., Chilliard, Y., Delavaud, C., Gerritsen, R., van der Lende, T., 2003.*** Association of leptin gene polymorphisms with serum leptin concentration in dairy cows. Mammalian Genome. 14, 657–663.
- ***Liefers, S. C., Veerkamp, R. F., Pas, M. F. W. T., Delavaud, C., Chilliard, Y., Platje, M., van der Lende, T., 2005.*** Leptin promoter mutations affect leptin levels and performance traits in dairy cows. Animal Genetics. 36, 111-118.
- ***Lin, S. L., Miller, J. D., Ying, S. Y., 2006.*** Intronic microRNA (miRNA). J Biomed Biotechnol. 2006, 26818.

- ***Lin, Y., Li, Q., 2005.*** The Regulation of Development and Lactation of the Mammary Gland by Leptin. The Journal of American Science. . 1, 63-67.

- ***Marai, I. F. M., Daader, A. H., A.M., S., El-Menshawy, S. M. S., 2009.*** Non-genetic factors affecting growth and reproduction traits of buffaloes under dry management housing (in sub-tropical environment) in Egypt. Livestock Research for Rural Development. 21.

- ***Mattick, J. S., Gagen, M. J., 2001.*** The evolution of controlled multitasked gene networks: the role of introns and other noncoding RNAs in the development of complex organisms. Mol Biol Evol. 18, 1611-30.

- ***Miglior, F., Sewalem, A., Jamrozik, J., Bohmanova, J., Lefebvre, D. M., Moore, R. K., 2007.*** Genetic analysis of milk urea nitrogen and lactose and their relationships with other production traits in Canadian Holstein cattle. Journal of Dairy Science. 90, 2468-2479.

- ***Neville, M. C., McFadden, T. B., Forsyth, I., 2002.*** Hormonal regulation of mammary differentiation and milk secretion. Journal of Mammary Gland Biology and Neoplasia. 7, 49-66.

- ***Nkrumah, J. D., C, L., Basarab, J. A., Guercio, S., Meng, Y., Murdoch, B., Hansen, C., Moore, S. S., 2004.*** Association of a single nucleotide polymorphism in the bovine leptin gene with feed intake, growth, feed efficiency, feeding behavior and carcass merit. Journal of Animal Science . 84, 211–219.

- ***Orru, L., Terzano, G. M., Napolitano, F., Savarese, M. C., De Matteis, G., Scata, M. C., Catillo, G., Moioli, B., 2007.*** DNA Polymorphisms in River Buffalo Leptin Gene. Italian Journal of Animal Science. 6, 342-344.

- ***Sadeghi, M., Moradi Shahr Babak, M., Rahimi, G., Nejati Javaremi, A., 2008.*** Effect of leptin gene polymorphism on the breeding value of milk production traits in Iranian Holstein. Animal 2, 999–1002.

- ***Sayed-Ahmed, A., Elmorsy, S. E., Rudas, P., Bartha, T., 2003.*** Partial cloning and localization of leptin and leptin receptor in the mammary gland of the Egyptian water buffalo. Domest Anim Endocrinol. 25, 303-14.

- ***Schenkel, F. S., Miller, S. P., Ye, X., Moore, S. S., Nkrumah, J. D., Li, C., Yu, J., Mandell, I. B., Wilton, J. W., Williams, J. L., 2005.*** Association of single nucleotide polymorphisms in the leptin gene with carcass and meat quality traits of beef cattle. Journal of Animal Science . 83, 2009–2020.

- ***Smith-Kirwin, S. M., O'Connor, D. M., Johnston, J., De Lancey, E., Hassink, S. G., Funanage, V. L., 1998.*** Leptin expression in human mammary epithelial cells and breast milk. Journal of Clinical Endocrinology and Metabolism. 83, 1810-1813.

- ***Smith, S. J., Cases, S., Jensen, D. R., Chen, H. C., Sande, E., Tow, B., Sanan, D. A., Raber, J., Eckel, R. H., Farese, R. V., 2000.*** Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. Nature Genetics. 25, 87-90.

- ***Stone, R. T., Kappes, S. M., Beattie, C. W., 1996.*** The bovine homolog of the obese gene maps to Chromosome 4. Mammalian Genome. 7, 399-400.

- ***Vignal, A., Milan, D., SanCristobal, M., Eggen, A., 2002.*** A review on SNP and other types of molecular markers and their use in animal genetics. Genetics Selection Evolution. 34, 275-305.