A NOVEL SNP (C.258+43C>T) IN LPL GENE AND ASSOCIATION WITH MILK PRODUCTION IN BUFFALOES

Akhtar Ali[1,*], Zukhruf Baig[2], Masroor Ellahi Babar[1], Asif Nadeem[2], Tanveer Hussain[1], Rashid Saif[1] and Maryam Javed[2]

ABSTRACT

Buffalo milk is the most preferred commodity in Pakistan due to high fat contents. Hydrolysis of circulating triglycerides and uptake of fatty acids in the mammary gland is regulated by lipoprotein lipase (LPL). T his study is designed to identify single nucleotide polymorphisms (SNPs) in LPL gene among high and low milk producing buffalo breeds of Pakistan. We selected samples (n = 50) of each Nili-Ravi a high milk producing and Azakheli a low milk producing buffalo breeds. Blood samples were collected for DNA extraction. LPL region of exon 2 region along with exon/ intron boundaries were sequenced and data was analyzed for variation detection. Allele frequency was calculated using Hardy-Weinberg equation and in-silico analysis was performed for functional prediction and genetic diversity assessment. We found one single nucleotide polymorphism c.258+43C>T in the intronic region of LPL gene. This polymorphism followed the Hardy-Weinberg equilibrium in both Nili-Ravi (P-value = 0.538) and Azakheli (P-value = 0.077). Association of T-allele analysis predicted it significantly higher (P-value = 0.009) in Nili-Ravi. We found one novel SNP (c.258+43C>T) in the LPL gene in both high and low milk producing buffalo breeds of Pakistan but high in Nili-Ravi. Lower splice site effect suggests its less strength of association with milk producing trait.

Keywords: *Bubalus bubalis*, buffaloes, LPL gene, Azakheli, Nili-Ravi, polymorphism, genetic diversity

INTRODUCTION

Pakistan is an agriculture country and the second largest buffalo milk producing in the world. Buffalo population is about 33.7 million and yields 30,462 and 1,829 tons of milk and meat respectively (FAO, 2016). Five known buffalo breeds Nili, Ravi, Nili-Ravi, Kundi and Azakheli are found in Pakistan. But the most common are Nili-Ravi and Kundhi breeds while a sizeable number of Azakheli, Nili and Ravi also exist. Nili-Ravi considered as juncture of Nili and Ravi breed is known as high milk producing with about 2300 liters per lactation and populated in the riverine area of Punjab province (Sajid *et al.*, 2010). While Azakheli as low milk producing with 1800 liters milk yield per lactation and is limited to Swat valley of KPK province (Khan *et al.*, 2007).

Buffalo milk is the second largest

[1]Faculty of Science and Technology, Virtual University of Pakistan, Lahore, Pakistan, *E-mail: drakhtar.ali@hotmail.com

[2]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan

produced milk in the world and is of nutritionally important in various parts of the world. It is the preferred species due to significantly higher levels of fat contents compared to cow's milk (Ménard *et al*., 2010). Milk fat is predominantly consists of triglycerides (TGs) and its synthesis correlates with milk production and composition (Lock and Bauman, 2004). Lipoprotein lipase (LPL) is an enzyme produced by the parenchymal cells and localized in skeletal muscles and white adipose tissues. It plays a vital role in TGs metabolism. LPL contributes important role in fat metabolism, lipid transportation, fat deposition and regulates the energy balance (Wang *et al*., 2012). Expression of LPL gene increases significantly in the lactating compared to non-lactating animals (Yadav *et al*., 2012). LPL molecule is a 478 amino acid long polypeptide having two domains which exhibit the normal hydrolytic activity. These are the amino terminal and carboxyl terminal domains connected with a flexible peptide chain (Wong *et al*., 1994). The carboxyl domain contains heparin-binding region responsible for lipoprotein binding and amino terminal has catalytic site Ser 132, Asp156 and His 241 which hydrolyze the triglycerides into free fatty acids and monoacylglycerols. This catalytic center is encoded by exon2 of LPL gene located at 8p22 position. Expression of LPL increases in mammary glands during lactation which suggests its possible role in milk trait.

We aimed to access the genetic variants in exon2 of LPL gene in Nili-Ravi buffalo, a high milk producing and Azakheli a low milk producing buffalo breed of Pakistan.

MATERIALS AND METHODS

Sample selection

We selected randomly (n = 50) animals of each Azakheli from Swat, KPK and Nili-Ravi from Buffalo Research Institute, Pattoki, Punjab. Blood samples were collected (3 to 5 mL) through venipuncture from Jugular vein. Blood samples were preserved in vacutainer containing 0.5 M ethylene diamine tetra acetic acid as an anticoagulant. This study was approved from ethical committee.

DNA extraction and amplification

Genomic DNA from white blood cells was extracted by standard inorganic method with some modifications (Sambrook and Russell, 2001). DNA was identified on 0.8% agarose gel stained with Ethidium Bromide and quantified using Nano Drop. Primer used was designed with Primer3 software (Koressaar and Remm, 2007) as shown in Table 1.

PCR reaction mixture of 25 µL containing 2.0 µL of DNA (25 ng/µL), 2.0 µL of dNTPs (2.5 M), 0.2 µL of Taq. polymerase (5 U/µL), 2.5 µL of $MgCl_2$, 2.0 µL of PCR buffer, and 1.0 µL of each forward and reverse primer (10 pmol/µL). Amplification was performed by initial denaturation of template DNA at 95°C for 5 minutes followed by cyclic denaturation at 94°C for 30 seconds, annealing temperature at 55°C for 30 seconds, extension at 72°C for 40 seconds and a final extension at 72°C for 10 minutes were carried out in thermocycler (Bio-Rad, USA). Amplicons were run on 1.2% agarose gel for confirmation.

Sequencing and alignment

PCR product was precipitated with 64% ethanol

and sequenced using genetic analyzer ABI-3130xl. Sequence data was aligned using Bio-Edit and online blast2 alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Single nucleotide variations were identified.

Statistical and phylogenetic analysis

Chi-square test was applied for allele frequency distribution and calculation of association with milk producing trait. Splicing effect was predicted using ESE software version 2.0. Phylogenetic tree was constructed using the available sequence of *Bos taurus*.

RESULTS AND DISCUSSION

Sequence analysis showed one single base change C>T in the second intron of LPL gene at position c.258+43 in both buffalo breeds (Figure 1). This single nucleotide polymorphism (SNP) was observed as heterozygous and homozygous in Azakheli while in Nili-Ravi the wild type was only found as heterozygous. Allele frequency distribution followed the Hardy-Weinberg equilibrium (Table 2).

Exon splicing effect of c.258+43C>T was predicted using ESE finder version 2.0 (Cartegni *et al*., 2003) Threshold value was 2.676 and variant at c.258+43C>T was predicted to be less probability of effecting the splicing with score 1.62424 below than the threshold value (Figure 2).

Phylogenetic tree constructed using MEGA 7.0 software to assess the evolutionary relationship with Bos Taurus (Figure 3). Branch length of 0.0017 million years shows their shared ancestor (Kumar *et al*., 2016).

LPL enzyme is a glycoprotein that plays a central role in plasma triglyceride metabolism by hydrolyzing triglyceride-rich chylomicrons and very low density lipoproteins. Sequenced data of lipoprotein lipase (LPL) gene showed only one base change in c.258+43C>T in Azakheli and Nili-Ravi buffalo breeds.

We found one SNP in the intronic region of LPL gene but with a significantly increased frequency p-value in high milk producing breed. Most of eukaryotic mRNAs are spliced to remove intron. This splicing generates uninterrupted frames that can be translated into polypeptide molecules. Splicing mechanism is highly regulated and generate alternate splice form that code for protein. Splicing is regulated by binding of RNA-binding proteins with specific sequences of mRNA (Xiong *et al*., 2015). There are many traits associated with altered splicing. Splicing effect of this SNP is predicted as 1.624 below to the threshold value. LPL is well versed in human metabolic diseases but less extensively studied in dairy animals particularly *Bubalus bubalis*. LPL gene studied in cattle breed identified six novel SNPs, one SNP associated with growth traits (Wang *et al*., 2012). Complete coding sequence of LPL gene showed two polymorphisms associated with milk trait in goat. One missense in the signal peptide c.50G>C region associated with milk fat contents and tend to affect the dry milk weight (Badaoui *et al*., 2007). Other milk production trait gene like β4GaT-1 is reported as polymorphic in Nili-Ravi buffalo (Sohail *et al*., 2016).

Yak LPL shares about 99.5% nucleotide sequence and 100% amino acid sequence similarity with *Bos Taurus*. A single base change C>T in 7th exon of LPL gene investigated in the yak (*Bos grunniens*) associated with carcass trait and visceral fat deposition in the yak (Ding *et al*., 2012). We could not find any other genetic variant in the LPL gene due to its conserved nature. The exon 2

Table 1. Primer pair used for sequencing of exonic region of LPL gene.

Primer-ID	Sequence	GC%	Tm	Product
LPL-1	5-CACCCTCTAATTAACCTCCTATCC-3	45.8	58.7	357
LPL-2	5- GATTCCTCAATCCCCACCT-3	52.6	58.7	

Table 2. Allele frequency distribution in Nili-Ravi and Azakheli buffalo [HWE: Hardy-Weinberg equilibrium].

Breed	CC	CT	TT	Allele Frequency		HWE *p*-value	Significance level
				p	Q		
Azakheli	4	12	34	0.200	0.800	0.077	Not significant
Nili-Ravi	0	8	42	0.080	0.920	0.538	Not significant

The allele frequency results found significant higher p-value 0.009 of c.258+43 C>T polymorphism in Nili-Ravie compared to Azakheli.

Table 3. Chi-square calculator used for contingency table for allele frequency comparison in Azakheli and Nili-Ravi buffalo [whereas () is the expected cell totals and [ ] the chi-square statistic for each cell].

	Azakheli	Nili-Ravi	Totals	Significance level (p-value)
C-allele	20 (13.69) [2.91]	8 (14.31) [2.78]	28	Significant (0.009)
T-allele	68 (74.31) [0.54]	84 (77.69) [0.51]	152	
Totals	88	92	180	

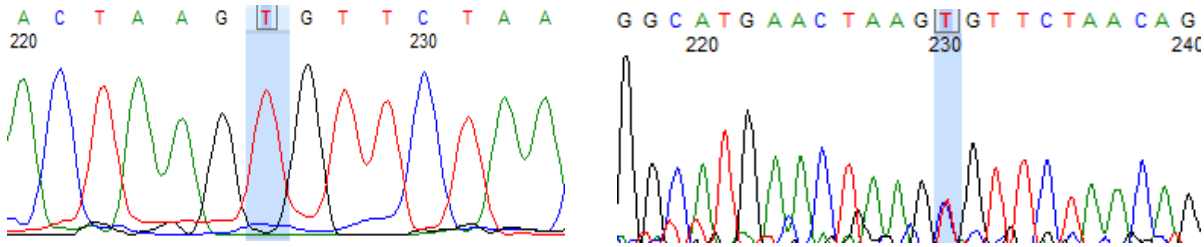

Figure 1. LPL gene SNP (c.258+43 C>T) heterozygous (a) and homozygous (b) form in Azakheli buffalo.

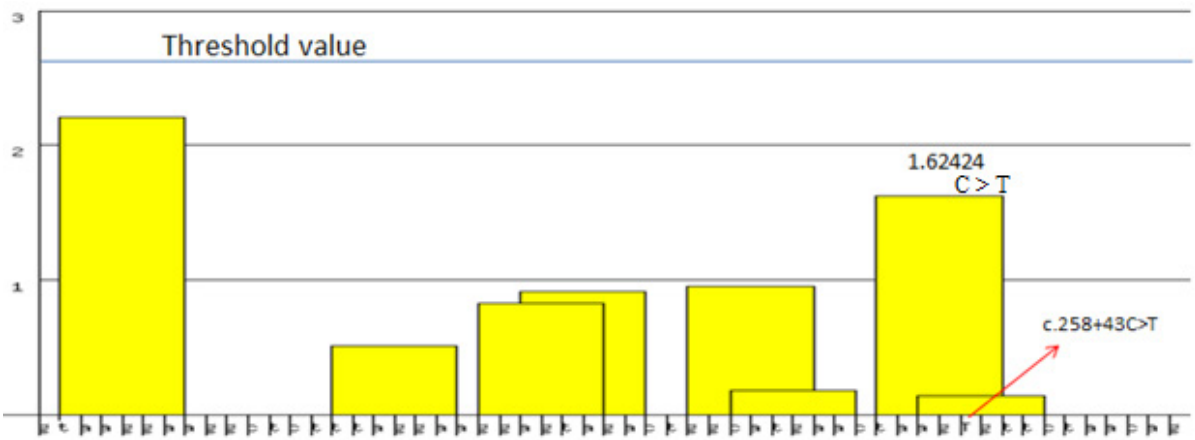

Figure 2. Graphical presentation of c.258+43C>T in LPL gene in bovine.

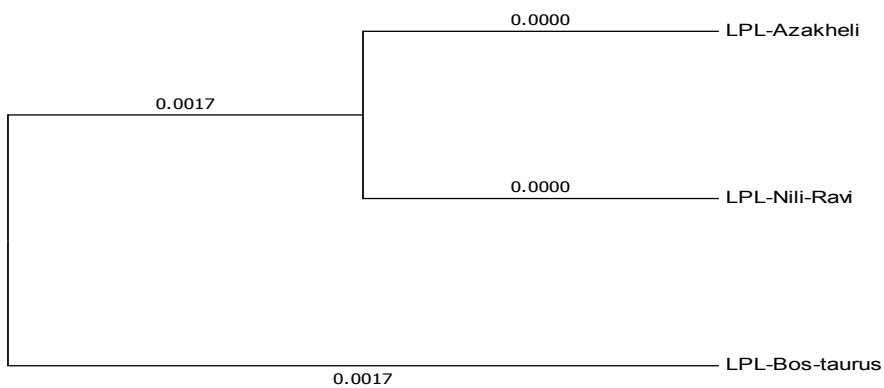

Figure 3. Phylogenetic tree constructed for LPL gene using MEGA 7.0 software.

exhibits a conserved sequence in all mammalian species (Murthy *et al*., 1996). A highly conserved structure and function is shown by the LPL gene among some animals, which defended the assessment of the LPL gene in the buffalo. Genetic variations in traits can serve as the basis for future breeding programs in dairy animals. Based upon our findings, LPL can be investigated on large scale for growth as well as milk production trait and it can be a useful genetic marker for bovine reproduction and breeding programs.

CONCLUSION

A single nucleotide polymorphism c.258+43C>T identified in both high and low milk producing buffalo breeds of Pakistan with significantly higher level of T-allele in Nili-Ravi. To date, this polymorphism is not reported in the buffalo but due to relatively less splice site effect it depicts low association with milk producing trait.

ACKNOWLEDGEMENT

Higher Education Commission Pakistan and Buffalo Research Institute, Pattoki are highly acknowledged for their support. Dr. Habib-un-Nabi is also appreciated for his support in sampling from Swat valley.

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