Sequencing of lipoprotein lipase gene in the Mediterranean river buffalo identified novel variants affecting gene expression

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ABSTRACT

Lipoprotein lipase (LPL) is a key enzyme for lipid metabolism, playing a fundamental role in the composition of fat in adipose tissue and milk. The *LPL* gene has been seldom investigated in dairy ruminants and barely studied in river buffalo (*Bubalus bubalis*). The aim of this work was to explore the genetic diversity of *LPL* and its promoter and to identify functional mutations, using a combined approach based on sequencing, dual-color electrophoretic mobility shift assay, and quantitative PCR. Thirteen consensus sequences for transcription factors were found in the promoter. Eleven SNP were detected, and the attention was focused on the SNP with potential functional effects: g.-446A>G, because the presence of G created a consensus motif for the transcription factor Sp1, and g.107A>G, which was the only exonic SNP. We developed PCR-RFLP methods for genotyping the 2 SNP and calculated the allele frequencies. A strong linkage disequilibrium ($D' = 1$; $r^2 = 0.903$) was found between the 2 SNP. The dual-color electrophoretic mobility shift assay demonstrated that only genotype g.-446GG allowed the binding of the Sp1 transcription factor, resulting in overexpression of the gene (~2.5 fold), as confirmed by the quantitative PCR results. Haploinsufficiency is proposed as a regulation mechanism. This study adds further knowledge on the structure of the *LPL* gene and its expression in river buffalo, with potential effects on milk qualitative and quantitative production.

Key words: Mediterranean river buffalo, *LPL* gene, promoter, gene expression, Sp1 transcription factor

INTRODUCTION

Milk fat is mostly composed of triglycerides (over 95%), whose fatty acids (FA) can be originated by ruminal fermentation of carbohydrates (de novo synthesis) or come directly from the plasma by the action of lipoprotein lipase (LPL; Barber et al., 1997).

Lipoprotein lipase is the key enzyme involved in the metabolism, transport, and regulation of lipid in serum. It acts on the surface of the capillary endothelium by hydrolyzing triglycerides contained in circulating chylomicrons and very low density lipoprotein to provide free FA to tissues. Regulation of the *LPL* gene is very complex, and it responds in tissue-specific ways to dietary and hormonal changes and environmental conditions. Adipose and muscular tissues control the circulating levels of plasma lipoproteins; however, LPL is expressed in other sites, including the mammary gland, nervous system, heart, liver, pancreatic islet cells, and lungs (Merkel et al., 2002).

The *LPL* gene has been investigated in several species, and SNP have been reported in cattle (Tank and Pomp, 1994; Lien et al., 1995; Oh et al., 2013) and in yak (Ding et al., 2012), where *LPL* has been proposed as major candidate gene for lipid deposition in carcass and FA composition. Marchitelli et al. (2013) reported a significant effect of the *LPL* g.25732A allele on milk-fat percentage in the Piedmontese cattle breed.

In goats, sequencing of the *LPL* cDNA allowed the identification of a missense mutation associated with milk-fat content (Badaoui et al., 2007). In the same species, Crepaldi et al. (2013) found an association between the transversion C50G in exon 1 and milk yield, thus reporting for the first time an effect of *LPL* on this trait.

Among farm animals, river buffalo produce milk with the highest fat content, estimated at 8.0% (<http://www.anasb.it/home.htm>). Milk yield and composition are

Received November 27, 2019.

Accepted March 13, 2020.

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of great economic importance for the dairy industry in Italy, where this species is mainly reared for the production of mozzarella cheese (protected designation of origin, Reg. EC 510/2006). The increasing demand for this product in both national and international markets facilitated the development of the buffalo dairy industry, which currently includes 102 cheese factories and more than 20,000 operators, with a consolidated turnover of about €720 million (ISMEA, 2016). In this context, the discovery of genetic markers affecting milk yield and composition could contribute to the genetic improvement of the species.

The objectives of this study were to explore the genetic diversity of *LPL* gene in the Italian Mediterranean breed of river buffalo (*Bubalus bubalis*), to describe the main regulatory elements of the promoter, and to investigate possible effects of polymorphism on the gene expression.

MATERIALS AND METHODS

Sample Collection and Nucleic Acid Isolation

A total of 523 river buffalo reared in 14 farms located in the Campania region of Southern Italy were used in this study. Blood samples were collected from buffalo in collaboration with the Italian National Association of Buffalo Breeders, during routine prophylaxis of each farm. For this reason, animal care and use committee approval was not necessary. In addition, 6 individual milk samples were collected after genotyping at the SNP g.107A>G and g.-446A>G for the isolation of RNA to perform qualitative PCR.

Genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Total RNA was isolated from milk somatic cells by Tri-Reagent (Sigma-Aldrich, Milan, Italy), according to manufacturer guidelines. Traces of contaminating DNA were removed using DNase I treatment (Thermo Fisher Scientific, Waltham, MA).

Concentrations of DNA and RNA and optical density (OD) ratios of the samples were measured with the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

Sequencing and Bioinformatics

Genomic DNA of 14 randomly chosen individual samples was amplified by iCycler (Bio-Rad Laboratories, Hercules, CA), using primers designed from the buffalo genome sequence (European Molecular Biology Laboratory, Heidelberg, Germany, acc. no. AWWX01438720.1; Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17968>).

The PCR reaction mix (25 µL) included 50 ng of genomic DNA, 1× PCR Buffer (Promega, Madison, WI), 2.5 mM magnesium chloride, 5 pmol of each primer, 200 µM each dNTP, and 1 U of *Taq* DNA Polymerase (Promega). The thermal conditions were 95°C for 4 min and 35 cycles at 95°C for 45 s and annealing for 45 s, at temperatures depending on the amplicon (Supplemental Table S1), and extension at 72°C for 45 s. A final extension was carried out at 72°C for 5 min.

Product specificity was confirmed by electrophoresis in 1.5% agarose gel in 0.5× Tris-borate EDTA (TBE) buffer, stained with SYBR Green (Sigma-Aldrich, Saint Louis, MO). The PCR products were purified and sequenced on both strands at Microsynth (Vienna, Austria).

Homology search, comparison among sequences, and multiple alignment for SNP discovery were accomplished using DNAsis-MAX (Hitachi, Chiyoda City, Tokyo, Japan). Polymorphisms found were submitted to the European Variation Archive (BioProject Acc. No. PRJEB28835). The putative binding sites for transcription factor at the promoter were searched by Transfact 7.0 database under the most stringent condition of analysis (85% as minimum binding score and 100% similarity of the sequence to consensus matrix).

Genotyping of the SNPs

The entire panel of 523 animals was genotyped by Biosearch Technologies (Herts, UK) using a competitive allele-specific PCR system (KASPar) for the SNP g.107A>G. Also, a PCR-RFLP method was set up for the same SNP to facilitate future homemade genotyping. In brief, 10 µL of exon 1 amplicons from informative samples were digested with 1 µL of Fastdigest *DdeI* (5'-C↓TNAG-3') endonuclease (Thermo Fisher Scientific) for 10 min at 37°C, according to manufacturer guidelines.

Forty samples (2 or 3 samples from each farm, randomly chosen from the panel of 523 buffalo) were genotyped for the SNP g.-446A>G, located in the promoter by digesting the amplicon with the endonuclease *SsiI* (5'-C↓CGC-3') overnight at 37°C, in the same conditions described above.

Reference heterozygous controls were always included to guarantee the correct assignment of genotypes in each PCR-RFLP experiment. The digestion products were resolved on 2.5% agarose gel in 1× TBE buffer and stained with SYBR Green.

Dual-Color Electrophoretic Mobility Shift Assay

Single-stranded DNA probes (Table 1) corresponding to the nucleotides −458 to −434 of the *LPL* promoter

Table 1. Oligonucleotide primer sequences, positions, and applications¹

Application and gene	Region	Primer sequence (F, forward; R, reverse)		EMBL ID	Size (bp)	Endonuclease
Genotyping <i>LPL</i>	Promoter	F	5'-GCAAGTTCAAGCTGCAGAAA-3'	AWWX01438720.1	151	<i>Sst</i> I
		R	5'-ATTTGGGCAATACCCAGCTT-3'			
	Exon 1	F	5'-GGAAACCTGCCGCTTCTA-3'		200	<i>Dde</i> I
		R	5'-GCTCAGAGCCAGCAGAA-3'			
Real-time PCR	<i>LPL</i>	F	5'-GGAAACCTGCCGCTTCTA-3'	AWWX01438720.1	200	—
		R	5'-GCTCAGAGCCAGCAGAA-3'			
	<i>ACTB</i>	F	5'-TCCTCCCTGGAGAAGAGCTA-3'	NM_001290932	101	—
		R	5'-AGGAAGGAAGGCTGGAAGAG-3'			
	18S rRNA	F	5'-CGTTCTTAGTTGGTGG-3'	NR_036642	76	—
		R	5'-GTAAGTAGTTAGCATGC-3'			
EMSA ²		Genotype	Probe (5' to 3')			
<i>LPL</i>	Promoter	AA	5'-AGGAGAATTGGC <u>A</u> GGGGAGGGGGGG-3'	AWWX01438720.1	25	—
		GG	5'-AGGAGAATTGGC <u>G</u> GGGGAGGGGGGG-3'			

¹Primers were designed using DNAsis software (Hitachi Software Engineering Co., San Bruno, CA).

²EMSA = electrophoretic mobility shift assay.

were co-denatured at 95°C for 5 min in 0.2× saline sodium citrate buffer and annealed at room temperature for 2 h with their complementary sequence to obtain double-stranded probes (homozygous AA and GG). Concentrations and OD ratios were measured with the Nanodrop ND-1000 Spectrophotometer as reported previously, and dilutions were prepared to a final concentration of 80 ng/μL.

Electrophoretic mobility shift assay (EMSA) reactions were carried out according to Gu et al. (2019). Briefly, binding reactions were set up by incubating 0.5 band-forming units of Sp1 transcription factor (Enzo Life Science Inc., Farmingdale, NY) in 1× Enzo buffer with 40 ng of DNA probe. After incubation at 37°C for 20 min, the samples were loaded in 5% native polyacrylamide gel and run at 150 V for 90 min in prechilled TBE 0.5×, according to Jing et al. (2013). Staining was performed as sequential steps in dual color, SYBR Green for the DNA, and SYPRO Ruby for the protein, according to the manufacturer guidelines of the EMSA kit (Thermo Fisher Scientific). The image was acquired using the Gel Doc EZ System (Bio-Rad Laboratories) and the digital images pseudo-colored and overlaid.

Quantitative Real-Time PCR Analysis

Comparative expression of *LPL* was examined in 6 Mediterranean river buffalo chosen according to the genotypes at the SNP g.-446A>G and g.107A>G (2 samples double homozygous AA, 2 samples double heterozygous AG, and 2 samples double homozygous GG). The animals belonged to the same farm, and were in third lactation, at 210 ± 11 DIM, free from mastitis.

Reverse transcription was accomplished by Verso cDNA synthesis kit (Thermo Fisher Scientific) starting from 100 ng of total RNA in a final reaction volume of 20 μL, and using a mixture of oligo dT₁₈ and Random Hexamer as primers, according to the protocol suggested by the company.

Quantitative PCR was performed using 2× QuantiTect SYBR Green PCR Master Mix in presence of ROX passive reference dye (Qiagen, Hilden, Germany). Analysis was carried out with a Mini Opticon Real-time PCR System (Bio-Rad) for 40 cycles at 95°C for 15 s, 55°C for 15 s and 72°C for 20 s. Amplification specificity was checked using a melting curve, following the manufacturer instructions. Primers used for *LPL*, *ACTB*, and 18S rRNA expression are listed in Table 1. Two biological replicates per sample were analyzed, and each replicate was amplified in triplicate (technical replicates) according to Gu et al. (2019). Relative gene expression of *LPL* was determined using *ACTB* (β-actin) and 18S rRNA as endogenous controls using the geometric mean values for normalization. Results are reported as fold-change relative to the mean.

Statistical Analysis

Allelic frequencies were calculated by Popgene software (University of Alberta, Edmonton, Alberta, Canada). Haploview 4.2 was used to estimate the rate of linkage disequilibrium (D' and r^2) between the SNP g.107A>G and g.-446A>G. Unpaired Student's *t*-test was applied to evaluate the differences in the gene expression.

RESULTS AND DISCUSSION

The DNA regions covering the promoter, the 5'-untranslated region (5'-UTR), and all coding regions until the stop codon of the river buffalo *LPL* gene were amplified and sequenced, for a total of 5,510 bp (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17968>).

The analysis of the first 750 bp of the river buffalo *LPL* promoter greatly contributed to evaluating the factors involved in the regulation of the gene expression. In this respect, *LPL* has rarely been investigated in ruminants, with information limited to a short report in sheep (Volpe et al., 1994).

The bioinformatics analysis revealed 13 consensus sequences for transcription factors (Figure 1). Twelve of them were equally represented: 3 TATA boxes (−301/−306; −606/−611; −648/−653), 3 Oct-1 (−66/−75; −155/−164; −559/−608), 3 Sp1 (−3/+7; −6/−15; −444/−449) and 3 CCAAT enhancer binding protein alpha (C/EBPα) (−57/−64; −89/−95; −545/−551). In addition, a motif AP2 was found in position +34/+41.

Part of these regulatory elements have been described in humans, where transfection experiments in adipocytes identified proximal positive (−368 to −35) and distal negative (−724 to −565) *cis*-acting motifs (Previato et al., 1991). In particular, the proximal regulatory region was shown to contain multiple conserved binding sites including C/EBP and Oct-1.

The CCAAT enhancer binding proteins (C/EBP) are known to be crucial components of terminal adipocyte development and play, together with peroxisome proliferator activated receptor γ (PPAR γ), a master role in the regulation of adipogenesis in the network of transcription factors which interact at different stages of the process (Farmer, 2006). The *LPL* gene is one of the adipocyte-specific genes that are trans-activated by C/EBPα (Olofsson et al., 2008; Moseti et al., 2016). In river buffalo, C/EBPα motifs have been amply found in the promoter region of the stearyl-CoA desaturase gene, which is also involved in lipid metabolism and associated with milk-fat quality (Pauciullo et al., 2012).

Octamer binding protein 1 (Oct-1) belongs to the octamer-binding proteins, a group of highly conserved transcription factors that specifically bind to the octamer motif (ATGCAAAT) and to closely related sequences found in promoters and enhancers (Zhao, 2013). Octamer binding protein 1 regulates the expression of a variety of genes, including κ - (Pauciullo et al., 2013) and β -casein genes (Zhao et al., 2002, Pauciullo et al., 2014), oxytocin (Cosenza et al., 2017), and immunoglobulin genes (Dreyfus et al., 1987). Furthermore, Oct-1 is not a strong transcriptional activator by itself,

but works in conjunction with other co-activators as TATA-binding proteins (Zwilling et al., 1994).

In humans, Oct-1 plays a major role in the modulation of *LPL* gene expression with 3 elements involved, 1 distal and 2 proximal (Previato et al., 1991). In fact, deletion of the region containing the distal octamer sequence resulted in a 75 to 100% increase in activity. Conversely, the deletion of the region with the 2 proximal octamers resulted in the decrease of transcription activity of approximately 65% (Previato et al., 1991). Human octamer motifs are conserved in mouse and chicken *LPL* promoters (Yang et al., 1995) and homology analysis with buffalo promoter confirmed a similarity of 96%. Therefore, it is reasonable to suggest that the presence of conserved Oct-1 motifs leads to similar regulation mechanisms of *LPL* gene expression mediated by this transcription factor.

Two *cis*-acting regions of the human *LPL* promoter, known as LP-α (GCGACTATCTTCTTTCACCTAT-CATAACTCAATACGG) and LP-β (GGACGCAATGTGTGTCCCTCTATCCCTACATTGACTTTGC), are also considered important for transcriptional activation of the gene during the adipocyte differentiation (Enerbäck et al., 1992). The comparative analysis of the LP-α and LP-β consensus sequences with the reference representative genomes available and annotated for the *LPL* showed that the human LP-α sequence was found across many species (with different level of homology), including river buffalo with 85% of similarity (Figure 1). Conversely, no significant similarity was found in buffalo for the LP-β, which is present only in primates, in the sea otter, and in the Hawaiian monk seal (similarity ranging from 85 to 100%). This result suggests a major effect of LP-α on *LPL* regulation among different species. Further studies will be necessary to evaluate the real influence of this element on the transcriptional regulation of *LPL* in river buffalo.

The sequence analysis revealed 11 SNP in total (Table 2). Although the sequenced region covered only 20.3% of the *LPL* (taking as reference the bovine gene sequence NC_037335.1 and the buffalo genome AWWX01438720.1), this gene showed variability in the river buffalo. Such variability has been observed for other ruminants (Badaoui et al., 2007; Ding et al., 2012; Crepaldi et al., 2013; Oh et al., 2013) and humans, where about 100 polymorphisms have been identified in the *LPL* gene, mostly associated with the occurrence of familial hyperlipidemia and atherosclerosis due to a reduction or loss of *LPL* function (Merkel et al., 2002).

Six polymorphic sites were detected in intron 3. None of them affected key sites of the spliceosome machinery (e.g., acceptor sites, branch points, poly-pyrimidine tracts, donor sites). Therefore, these mutations are not expected to influence the gene expression.

Figure 1. Key regulatory motifs found in the 5' promoter region of the river buffalo *LPL* gene. Numbering is relative to the first nucleotide of the first exon (+1). The 5' untranslated region (UTR) of the exon 1 is reported in italic lowercase letters, whereas the coding region is in uppercase letters. Genetic diversity is indicated in bold (w = a/t; r = a/g; y = c/t).

The other 5 SNP were observed in the promoter (4 SNP) and in exon 1 (1 SNP). Of these, 2 are of special interest for their potential functional effects. The mutation g.-446A>G falls within the consensus sequence of an Sp1 transcription factor, which also regulates the expression of other lipogenic genes in river buffalo

such as the *SCD* (stearoyl-CoA desaturase) gene, as recently demonstrated by Gu et al. (2019). Conversely, the exonic mutation (g.107G>A) falls in the 5'-UTR, which is a noncoding region that plays an important role in translational regulation. In fact, in addition to consensus sequences for transcription factors, it con-

Figure 2. (A) Genotyping of the SNP g.107A>G in the exon 1 of river buffalo *LPL* by *DdeI* PCR-RFLP. Lane 1, GG homozygous sample; lane 2, heterozygous sample; lane 3, AA homozygous sample; lane 4, GeneRuler 50 bp DNA Ladder (Thermo Fisher Scientific, Waltham, MA); lane 5, undigested PCR product. The 5-bp band was not visible on the gel. (B) Genotyping of the SNP g.-446A>G in the *LPL* promoter by *SsiI* PCR-RFLP. Lane 6, AA homozygous sample; lane 7, heterozygous sample; lane 8, GG homozygous sample; lane 9, GeneRuler 50 bp DNA Ladder (Thermo Fisher Scientific); lane 10, undigested PCR product.

tains Kozak consensus sequences and *cis*-acting regulatory elements known as upstream open reading frames (Wethmar et al., 2010).

The PCR-RFLP protocols set up for the SNP genotyping proved to be effective for the allele discrimination. For g.107G>A, digestion of the PCR product (200 bp) with the *DdeI* endonuclease produced 2 fragments (195 and 5 bp) for the G allele, and 3 fragments (103, 92, and 5 bp) for the A allele (Figure 2A). For g.-446A>G, using the endonuclease *SsiI*, allele A remained undigested (151 bp), whereas allele G was restricted into 2 fragments (84 and 67 bp; Figure 2B).

Frequencies of 0.63 and 0.68 were calculated for the alleles g.107G and g.-446G respectively. At both SNPs, the genotype frequencies were in Hardy-Weinberg equilibrium (Table 3).

The allele frequency for the SNP g.107A>G in the sample of 40 animals used to genotype for the SNP g.-446A>G were very similar to those estimated from 523 animals. Therefore, we expected that the allele frequency did not change significantly in a larger sample also for the SNP g.-446A>G. Furthermore, strong linkage disequilibrium ($D' = 1$) was found between these 2 SNPs, and the high level of r^2 (0.903) allows the imputation of an allele at one SNP, known the allele at the other SNP.

To investigate the effects of the SNP g.-446A>G on the *LPL* expression, we tested in vitro whether a genotype-dependent binding reaction exists between the transcription factor Sp1 and this genetic marker, using EMSA. This technique is a powerful tool to assess the selective binding of a protein to a double-stranded DNA. The specific protein-DNA interaction results in the reduced migration rate of the complex

compared with the free DNA (Garner and Revzin, 1981). Therefore, dual-color EMSA experiments were performed using specific double-stranded DNA constructs (green color) corresponding to homozygous genotypes (g.-446AA and g.-446GG), each treated with a recombinant Sp1 transcription factor (red color). The presence of the dual-color band (yellow) and the shift in the mobility of the DNA construct (Figure 3) demonstrated that Sp1 interacted only with the genotype g.-446GG, generating a dual-color band (yellow) and a shift in the molecular weight of the DNA construct (Figure 3) confirming, *de facto*, the bioinformatics analysis (Figure 1). The Sp1 transcription factors are considered enhancer motifs for the basal expression of many genes, including *LPL*, whose transcription in hu-

Table 2. Polymorphisms found in the river buffalo *LPL* gene¹

Location	Position ²	Position ³	Polymorphism
Promoter	-517	13,606	A/T
	-446	13,677	A/G
	-433	13,690	A/G
	-358	13,765	C/T
Exon 1	+107	14,229	A/G
Intron 3	+33	27,206	A/G
	+38	27,211	A/G
	+43	27,216	T/G
	+64	27,237	A/G
	+70	27,243	T/A
	+149	27,322	A/G

¹Polymorphisms were submitted to the European Variation Archive (BioProject Acc. No. PRJEB28835).

²Numbering refers to each location and restarts from +1 (exon 1 and intron 3) or -1 (promoter) each time.

³Numbering refers to the buffalo genome sequence (EMBL ID: AWWX01438720.1).

Table 3. Absolute (relative) genotype frequencies and allele frequencies of Italian Mediterranean buffalo for the SNP g.107A>G and g.-446A>G at the *LPL* gene¹

SNP	Genotype at river buffalo <i>LPL</i>				Allele frequency	
	GG	AG	AA	Total	G	A
g.107A>G	217 (41.49)	228 (43.59)	78 (14.82)	523 (100)	0.63	0.37
g.-446A>G	18 (45.00)	19 (47.50)	3 (7.50)	40 (100)	0.68	0.32

¹No departure from Hardy-Weinberg equilibrium was observed in the population for both SNP ($\chi^2 = 1.999$ and $\chi^2 = 0.444$).

man is stimulated by this transcription factor itself or by a synergistic interaction between Sp1 and SREBP-1 (Yang and Deeb, 1998).

The exclusive interaction of the transcription factor Sp1 with the promoter having genotype g.-446GG led us to investigate also the effect of the SNP on the *LPL* transcription level. The q-PCR showed a significant overexpression of the genotype GG (~2.5-fold higher) compared with both AA ($P = 0.0019$) and AG ($P = 0.0017$) genotypes (Figure 4). Intermediate relative expression was expected for the AG genotype, as normally occurs in case of codominant alleles. However, AG had a relative expression similar to AA, indicating a condition compatible with complete dominance. Dominant mutations showing reduced gene dosage, expression, or protein activity are reported as haploinsufficiency, and loss of function is explained by mutations in promoters (Wilkie, 1994). Among genes showing haploinsufficiency, the low density lipoprotein receptor, whose activity is mediated by LPL, was reported as an example of autosomal dominant inheritance in very early studies (Sing et al., 1974; Goldstein et al., 1976; Wilkie, 1994; Dammerman and Breslow, 1995); LPL deficiency itself was reported as an autosomal recessive disorder (Babirak et al., 1989; Dammerman and Breslow, 1995). These studies support a possible dominance/recessive model also for the buffalo *LPL*. In particular, the dominant allele A covers the expression of the allele G in the heterozygous genotype, explaining the similar expression pattern for the genotypes AA and AG.

The upregulation of *LPL* as result of a polymorphism in the gene promoter has been reported also in human, where a T to G substitution at position -89 changed a CT element into a canonical Sp1-binding motif. The activity of this promoter variant assessed by transfection luciferase assays and EMSA was 129% compared with the wild-type ($P = 0.04$, t -test; Yang and Deeb, 1998). Similarly, Irvine et al. (2005) found that different mutations of the Sp1 sites have marked effects on *LPL* promoter activity with consequences on its expression. In river buffalo, the polymorphism g.-446A>G created a consensus sequence for the Sp1 binding element as observed by EMSA, and the presence of the recessive

genotype GG induced the Sp1 transcription factor to stimulate an additional *LPL* mRNA production as observed by the expression data.

Figure 3. Dual-color electromobility shift assay testing the interaction between the *LPL* promoter and the transcription factor Sp1. Lane 1, free DNA sample (green); lane 2, DNA probe with genotype AA (no binding); lane 3, DNA probe with genotype GG (binding and shift in yellow); lane 4, free Sp1 protein (red); lane 5, 20 bp DNA Ladder (Jena Bioscience, Jena, Germany).

Figure 4. Relative expression values (mean $\pm$ SEM) of *LPL* in 6 buffalo (2 animals for each genotype at g.107A>G and g.-446A>G). Unpaired Student's *t*-test was used to assess the differences among the genotypes (** $P < 0.01$).

These results could have practical implications for the improvement of milk quality. In fact, the upregulation of *LPL* in the mammary gland is mediated by the anterior pituitary through the release of prolactin (Thomson, 1992), in agreement with hormonal regulation of the mammary gland during the lactation period and nutrient transport supporting the milk production (Bionaz and Loor, 2008). In addition, the change in *LPL* expression in milk somatic cells may affect the natural function of LPL enzyme (i.e., the hydrolysis of FA from triglycerides and phospholipids in the milk).

Lipoprotein lipase regulates the hydrolysis of circulating triglycerides carried by chylomicrons and very low density lipoprotein and the uptake of FA by most tissues, including mammary gland and adipose tissues. In particular, LPL is critical for the uptake and secretion of the long-chain FA in milk (Iverson et al., 1995). In lactating females, LPL levels are almost completely depressed in adipose tissue, whereas levels in the mammary gland are high. As a consequence, circulating FA are directed to the mammary gland for milk-fat production (Iverson et al., 1995). Therefore, LPL may affect the qualitative and quantitative characteristics of milk fat through this mechanism. The effect of the *LPL* polymorphism on milk production in river buffalo is under investigation.

CONCLUSIONS

The genetic progress of Italian river buffalo focuses on the improvement of milk production traits; therefore, the genes regulating fat metabolism are important targets of study. In the present investigation, we provided

the first description of *LPL* promoter and reported on the genetic diversity found in that region and at exon level. The SNP g.-446A>G in the promoter region created a consensus sequence for the transcription factor Sp1, whose binding activity was demonstrated to be genotype-dependent. The specific bond to the GG genotype induced a significant increase in the *LPL* gene expression, which could be associated with the milk yield and composition. Furthermore, our results suggested a molecular model of haploinsufficiency that needs to be further investigated.

ACKNOWLEDGMENTS

The authors thank the buffalo owners in Italy for providing the samples, and the Italian National Association of Buffalo Breeders for helping in the collection. This work was financially supported by the project ProBufaLat (PAUA_RILO_17_01), Department of Agricultural, Forest and Food Sciences, University of Torino, Grugliasco (Italy). The authors confirm that they have no conflicts of interest.

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