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Full Length Research Paper

Two novel missense mutations in bovine *ATGL* gene and their association with economic traits in Qinchuan cattle

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Adipose triglyceride lipase (ATGL) as a triglyceride-specific lipase, plays a key role in the triglyceride liposis mobilization of fat tissue. In this study, based on the pyrosequencing technology, two novel missense mutations were identified, which were 3289 G>C in exon 6 bringing E277Q and 3514 A>T in exon 7 bringing T320S, respectively. The 3289 G>C mutation had significant effect on backfat thickness (BFT) (P < 0.01), and cattle with genotype CC had higher BFT than those with genotype GG and GC. The 3514 A>T locus was significantly associated with dressing percentage (DP), carcass chest depth (CCD) and marbling score (MS) (P < 0.05). Cattle with genotype TT had higher CCD and MS than those with genotype TT; cattle with genotype TT had higher DP than those with AA and AT. The results suggested that 3289 G>C and 3514 A>T SNPs of the *ATGL* gene may be useful as genetic markers for carcass and meat quality traits in cattle.

Key words: Adipose triglyceride lipase (ATGL) gene, polymorphism, pyrosequencing, meat quality, carcass traits.

INTRODUCTION

Studies on fat mobilization and deposition in bovine shows that a candidate gene approach, combining with several cases of quantitative trait locus (QTL) information, can be useful to identify DNA markers associated with fatness as well as other correlated traits (Schenkel et al., 2005; Rincker et al., 2006; Sellick et al., 2007).

Adipose triglyceride lipase (ATGL) plays an essential role in the control of fat cell lipolysis, which may further affect fat mobilization and deposition (Zimmermann et al., 2004; Smirnova et al., 2006). ATGL belongs to a protein family containing a patatin-like domain; it is a lipid hydrolase with an unusual folded topology that differs from classical lipases and selectively performs the first step in triacylglycerol hydrolysis, resulting in the formation of diacylglycerol and free fatty acid (Jenkins et al., 2004;Villena et al., 2004). ATGL protein is associated with lipid droplets and was found at a high level in adipose tissue and other tissues that have lipid stores (Lake et al., 2005). Its expression increases dramatically in the subcutaneous adipose development and maturation (Deiuliis et al., 2008; Li et al., 2009). Moreover, ATGL deficient mice showed blunted fat cell lipolysis and rapidly became obese (Haemmerle et al., 2006). The regulation of ATGL activity appears to be quite different when compared with hormone-sensitive lipase (HSL) (Schweiger et al., 2006). Unlike HSL, ATGL activity is not directly regulated at the post-translational level via protein kinase A (PKA)-mediated phosphorylation, but strongly stimulated by an activator protein annotated as α/β hydrolase domain containing protein 5 (Lass et al., 2006; Fischer et al., 2007; Miyoshi et al., 2007). In the previous study, a total of 12 polymorphisms were discovered in human *ATGL* gene, two SNPs and their haplotypes were significantly

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Table 1. Primers used for PCR amplification and pyrosequencing.

Primer	Sequence (5'-3')	Nucleotide position
ATGL1F[a]	AACCCCTTGCTGGCACTG	3232-3249
ATGL1R[a]	biotin-AGGTGGTCCTTCCCTCCAGTC	3315-3335
ATGL1S[b]	CCCGAGGATGCAGAT	3271-3285
ATGL2F[a]	CTGCTGGAGGCCTGCATG	3490-3507
ATGL2R[a]	biotin-GCAGCATGTTGGAGAGTGTGG	3527-3547
ATGL2S[b]	GGCCTGCATGGAGCC	3498-3512

[a]PCR primer used in the pyrosequencing; [b]Sequencing primer used in the pyrosequencing.

associated with plasma-free fatty acid concentration, triglyceride concentration, glucose level as well as risk of type2 diabetes (Schoenborn et al., 2006). Other researches showed that mutations in human *ATGL* gene were associated with functional defects of enzyme and accumulation of lipid in multiple tissues (Akiyama et al., 2007; Fischer et al., 2007).

As a crucial gene for fat mobilization and deposition, *ATGL* is suggested to be a candidate gene for fatty and meat quality traits in domestic animals. To date, no polymorphisms of the *ATGL* gene in bovine have been reported. Therefore, it is a preliminary and important work to analyze the genetic variations of *ATGL* gene in bovine. Here, we detected the polymorphisms within bovine *ATGL* gene, and assessed the association of polymorphisms in *ATGL* gene with carcass and meat quality traits. These will possibly contribute to animal breeding and genetics.

MATERIALS AND METHODS

DNA samples and data collection

A total of 702 adult cattle were randomly selected from breeding populations and used to analyze the *ATGL* allelic frequencies, including Qinchuan (QC, n = 432, Shaanxi province), Jiaxian red (JR, n = 72, Henan province), Nanyang (NY, n = 44, Henan province), Xia'nan (XN, n = 76, Henan province) and Luxi (LX, n = 78, Shandong province). The association study was performed in a total of 102 Qinchuan steers, which were castrated before sexual maturity and especially raised for beef. Carcass and meat quality traits were measured according to the criterion GB/T 17238-1998 Cutting Standard of Fresh and Chilled Beef in China (China Standard Publishing House). The following traits: live weight (LW), carcass weight (CW), dressing percentage (DP), carcass length (CL), carcass chest depth (CCD), hind leg length (HLL), backfat thickness (BFT), loin muscle area (LMA), marbling score (MS), water holding capacity (WHC) and meat tenderness (MT) were measured or calculated. DNA samples were extracted from leucocytes according to Mullenbach (Mullenbach et al., 1989).

Total RNA (from freezed fat tissues of 6 cattle of each breed) was isolated with the TrizolTM Reagent (Invitrogen). Quality and integrity of total RNA were detected by formamide denatureing gel, and concentration of total RNA was determined with a NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The first-strand cDNA was synthesized with 2 µg total RNA using PrimeScriptTM RT reagent Kit (TaKaRa, Dalian, China) with Oligo dT Primer.

PCR and conventional sequencing

According to the sequence of bovine *ATGL* gene (GenBank accession No. NW_001494551), a pair of primer (5'-CGA TGT TCC CCA AGG AGA CG-3' and 5'-GCC CTC CTC TGT GCA ACG AA-3') was designed to amplify a 1688bp product which contains the complete CDS region of bovine *ATGL* gene. After amplifying the cDNA pool of five breeds (Norton et al., 2004) respectively, the products were purified with Axygen kits (MBI fermentas, Vilnius, Lithuania) and the resulting fragments were analyzed on an automated sequencer (ABI PRISM 3730 DNA analyzer) to screen for the mutation sites. Genetic polymorphisms in the *ATGL* were identified and compared with each other using SeqMan (DNASTAR, Inc., Madison, WI, USA). Two mutations were discovered in the amplified region: 3289 G>C and 3514 A>T. Primers for pyrosequencing (Table 1) were designed by SNP Primer Design Pyrosequencing AB version 1.0.1 software, specifically to amplify regions flanking including the 3289 G>C and 3514 A>T, respectively.

Genotyping by pyrosequencing

Pyrosequencing was performed according to the instructions of the PSQ 96MA pyrosequencer manufacturer (Biotage AB, Uppsala, Sweden). The protocol consists of PCR amplification, preparation of single-stranded DNA from the PCR products, annealing of sequencing primer and real-time sequencing by the pyrosequencer. The antisense PCR primers were biotinylated to allow the immobilization of the PCR product on streptavidin-coated beads (Streptavidin Sepharose™ High Performance (Amersham Biosciences AB, Uppsala, Sweden), and single-stranded DNA was prepared using a pyrosequencing vacuum prep tool. The secondary structures were removed by denaturation at 80°C for 2 min. The annealing of the sequencing primer was performed at room temperature. Genotyping was performed on the automated PSQ 96MA 96-well pyrosequencer using the PSQ™ 96 SQA reagent kit (Biotage AB) according to the instructions of the manufacturer.

Statistical analysis

According to Nei methods (Nei and Roychoudhury, 1974), the following items, including genotypic frequencies, allelic frequencies, Hardy–Weinberg equilibriums, gene homozygosity, gene heterozygosity, effective allele numbers and polymorphism information

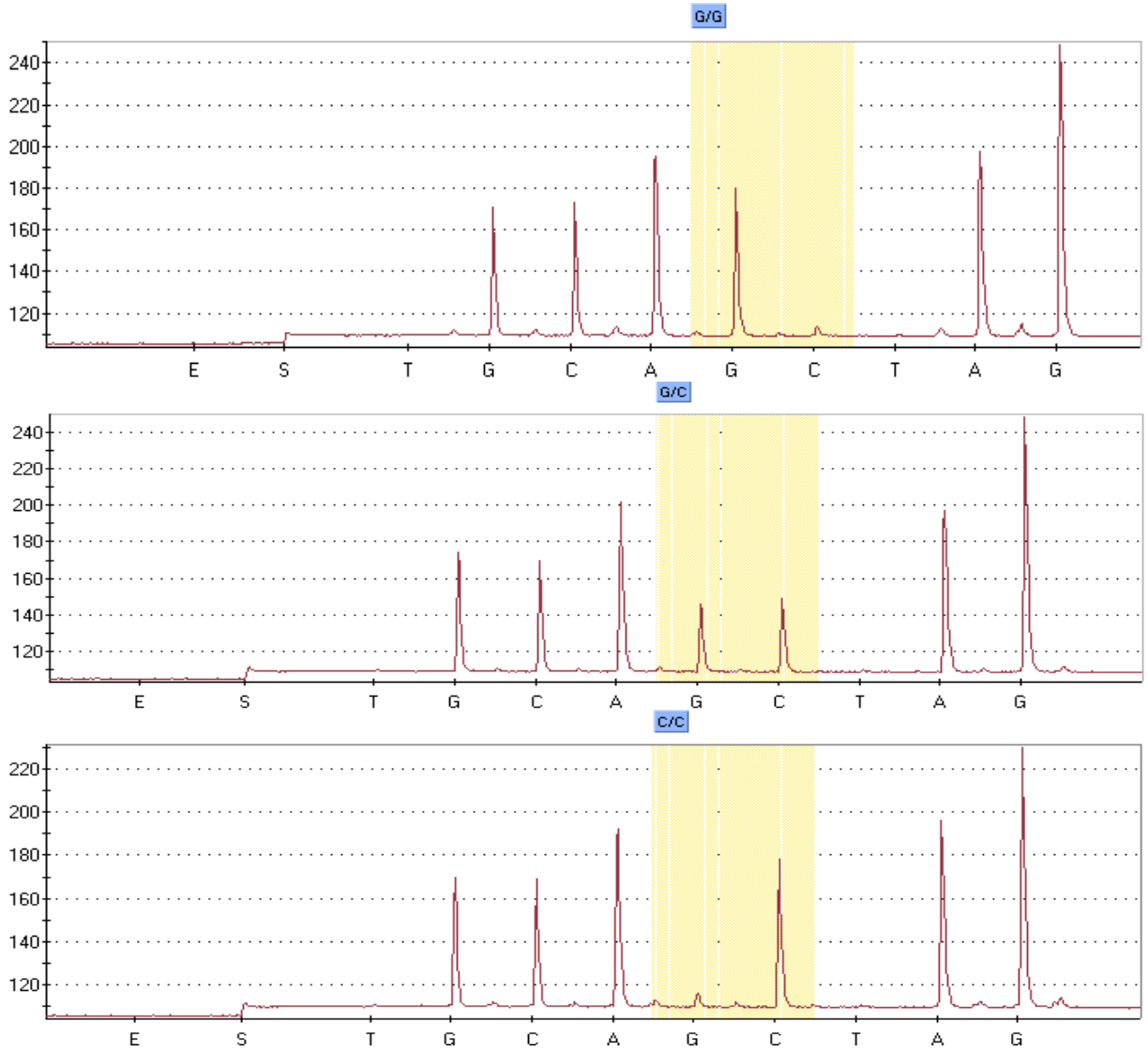

Figure 1. Results from three different SNP genotypes at 3289 G>C analyzed by pyrosequencing.

content (PIC), were calculated.

Statistical analysis of associations was performed between *ATGL* genotypes and carcass and meat quality traits of Qinchuan steers using SPSS (version 13.0). The model applied was:

$$Y_{ijk} = \mu + A_i + G_j + E_{ijk}$$

Y_{ijk}: stands for observed value; μ: overall mean for each trait; A_i: the fixed effect of *i*th age of slaughtering; G_j: the fixed *j*th genotype marker; E_{ijk}: random error.

RESULTS

In this study, a total of 2 SNPs in the entire coding region were identified, which were 3289 G>C (in exon6, p.E277Q) and 3514 A>T (in exon7, p.T320S) in *ATGL* gene. Five breeds summing up to 702 individuals were genotyped by pyrosequencing. We named the presence "G" as *ATGL*-G allele, the "C" as *ATGL*- C allele in the exon6 (Figure 1), the "A" as *ATGL*-A allele and "T" as *ATGL*-T allele in the exon7 (Figure 2).

Allele frequencies of loci 3289 G>C and 3514 A>T were investigated in five different beef breeds (Table 2). They were ranged from 0.7159 to 0.8229 and 0.7431 to 0.8977, respectively. Gene heterozygosity, effective allele numbers and PIC of the two SNPs were calculated (Table 3). According to the classification of PIC (low polymorphism if PIC value <0.25, median polymorphism if 0.25 < PIC value < 0.50, and high polymorphism if PIC > 0.50), all the beef population belong to the median polymorphism level at 3289 G>C locus except for QC population. JR, NY and XN belong to the low polymorphism level at 3514 A>T locus, while QC and LX belong

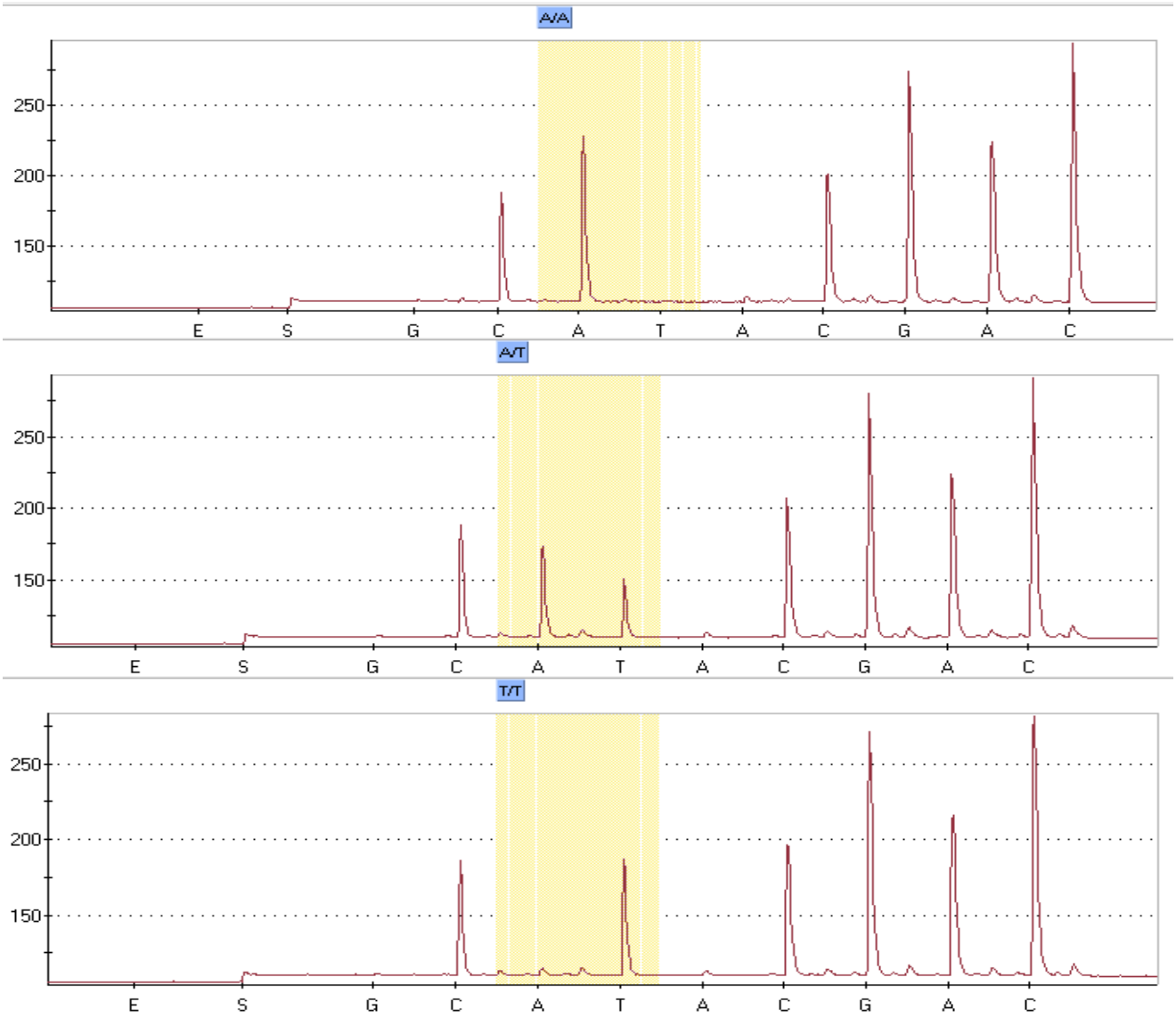

Figure 2. Results from three different SNP genotypes at 3514 A>T analyzed by pyrosequencing.

Table 2. Genotype frequencies and allelic frequencies of two mutations locus in bovine ATGL gene.

Breed	Genotype frequencies			Allele frequencies		χ2	Genotype frequencies			Allele frequencies		χ2
	GG	GC	CC	G	C		AA	AT	TT	A	T	
QC	0.7083	0.2292	0.0625	0.8229	0.1771	19.7286	0.5694	0.3472	0.0833	0.7431	0.2569	3.5522
JR	0.6389	0.3472	0.0139	0.8125	0.1875	1.4032	0.7361	0.2361	0.0278	0.8542	0.1458	0.1967
NY	0.5227	0.3864	0.0909	0.7159	0.2841	0.1107	0.7955	0.2045	0.0000	0.8977	0.1023	0.5711
XN	0.5263	0.3816	0.0921	0.7171	0.2829	0.2693	0.7105	0.2632	0.0263	0.8421	0.1579	0.0082
LX	0.5385	0.3590	0.1026	0.7179	0.2821	1.0072	0.6410	0.3205	0.0385	0.8013	0.1987	0.0032

to the median polymorphism level.

Further observations by a χ^2 test shows that genotypic frequencies are found to be significantly different in five breeds (χ^2 = 23.742, df = 8, p = 0.003 at the 3289 G>C locus; χ^2 = 21.369, df = 8, p = 0.006 at 3514 A>T locus, respectively) and allelic frequencies in analyzed populations are also found to be significantly different (χ^2 = 18.843, df = 4, p = 0.001 at the 3289 G>C locus, χ^2 =

Table 3. Genetic diversity at two mutations locus in the five populations.

Breed	3289 G>C				3514 A>T			
	Gene homozygosity	Gene heterozygosity	Effective allele number	PIC	Gene homozygosity	Gene heterozygosity	Effective allele number	PIC
QC	0.7086	0.2914	1.4113	0.2490	0.6182	0.3818	1.6177	0.3089
JR	0.6953	0.3047	1.4382	0.2583	0.7509	0.2491	1.3318	0.2181
NY	0.5932	0.4068	1.6857	0.3240	0.8164	0.1836	1.2249	0.1668
XN	0.5943	0.4057	1.6827	0.3234	0.7341	0.2659	1.3623	0.2306
LX	0.5950	0.4050	1.6807	0.3230	0.6815	0.3185	1.4673	0.2678

Table 4. X^2 and P values differences for genotypic and allelic frequencies among five cattle breeds at bovine ATGL 3289 G>C and 3514 A>T loci.

Loci	Breed	QC	JR	NY	XN	LX
3289 G>C	QC		6.547 (*P=0.038)	6.534 (*P=0.038)	9.948 (**P=0.007)	8.810 (*P=0.012)
	JR	0.091 (P=0.763)		4.494 (P=0.106)	5.111 (P=0.078)	5.565 (P=0.062)
	NY	6.016 (*P=0.014)	2.930 (P=0.087)		0.003 (P=0.999)	0.109 (P=0.947)
	XN	9.295 (**P=0.002)	3.727 (P=0.054)	0.000 (P=0.984)		0.107 (P=0.948)
	LX	9.343 (**P=0.002)	3.706 (P=0.054)	0.001 (P=0.973)	0.000 (P=0.987)	
3514 A>T	QC		7.715 (*P=0.021)	9.556 (**P=0.008)	6.352 (*P=0.042)	2.460 (P=0.292)
	JR	8.329 (**P=0.004)		1.470 (P=0.479)	0.145 (P=0.930)	1.574 (P=0.455)
	NY	10.397 (**P=0.001)	0.921 (P=0.337)		1.825 (P=0.402)	4.013 (P=0.134)
	XN	6.911 (**P=0.009)	0.337 (P=0.773)	1.454 (P=0.228)		0.884 (P=0.643)
	LX	2.402 (P=0.121)	1.462 (P=0.227)	3.818 (P=0.051)	0.875 (P=0.350)	

22.508, df = 4, p = 0.000 at 3514 A>T locus, respectively). Moreover, there were significant differences of genotypic frequencies between QC and JR, NY, XN and LX populations ($P < 0.05$ or $P < 0.01$), as well as significant differences of allelic frequencies between QC and NY, XN and LX populations at 3289 G>C locus ($P < 0.05$). At 3514 A>T locus, there were significant different genotypic frequencies and allelic frequencies between QC and JR, NY and XN populations (Table 4).

The establishment of relationships between genotypes and the 11 economic traits were attempted in 102 individuals (Tables 5 and 6). Cattle with the genotype CC had higher BFT than those with GG and GC ($P < 0.01$). Cattle with the genotype TT have higher CCD and MS than those with AA and cattle with the genotype TT have higher DP than those with AA and AT ($P < 0.05$).

DISCUSSION

Carcass and meat quality traits are controlled by polygenes with pleiotropic effect (Wang et al., 2005). SNP analysis is a well-established way to identify genes associated with traits of economic interest in livestock populations. For instance, the phenotypic variations are the key factor of mutations of muscle-related economic traits for the meat-production animals (Chan et al., 2009; Gill et al., 2009).

The bovine *ATGL* gene was located on chromosome 29 and organized by nine exons coding 486 amino acids. To our knowledge, this is the first report on the polymorphism of *ATGL* in the bovine. We detected the polymorphism of the entire coding region of the bovine *ATGL* gene, and revealed two novel SNPs that were confirmed in 702 cattle by direct PCR amplification and pyrosequencing. The 3289 G>C and 3514 A>T mutations were found in all analyzed breeds. The frequencies of mutant allele (C and T) were lower than wildtype allele (G and A) in all the experimental populations and this suggests that they might be recent mutations that have not spread so far. We did not find TT genotype in NY population. There are a couple of possibilities for this observation in the NY population, these include: (1) TT genotype possibly exists in the NY pedigrees population, but the individuals are not enough in our study, and (2) the animals with TT genotype do not exist in NY breed.

A number of studies were recently focused on the functional SNPs in *ATGL* gene in human. Fischer discovered four variations in coding regions of human *ATGL* gene including a C>T mutation at 584nt (causing substitution of P195L), a frame shift deletion at 808C (319Ter), a frame shift deletion at 847C (319Ter), and a nonsense mutation of C>T at 865nt (Q289Ter) in three myopathy patients with neutral lipid storage disease (Fischer et al., 2007).

Table 5. Association of 3289 G>C SNP genotypes with carcass and meat quality traits at bovine ATGL gene.

Traits	GG (Mean ± SD)	GC (Mean ± SD)	CC (Mean ± SD)	P-value
Live weight (LW) (kg)	502.29 ± 59.88	513.85 ± 62.97	478.25 ± 29.28	0.323
Carcass weight (CW) (kg)	266.23 ± 33.47	268.72 ± 45.58	255.20 ± 18.71	0.650
Dressing percentage (DP) (%)	0.53 ± 0.02	0.52 ± 0.02	0.53 ± 0.01	0.136
Carcass length (CL) (cm)	145.43 ± 7.63	147.85 ± 7.22	143.88 ± 4.76	0.264
Carcass chest depth (CCD) (cm)	29.35 ± 2.93	27.92 ± 3.68	29.75 ± 1.91	0.109
Hind leg length (HLL) (cm)	72.98 ± 3.83	72.76 ± 3.72	74.50 ± 1.93	0.495
Backfat thickness (BFT) (cm)	0.96 ± 0.25^{A}	1.04 ± 0.32^{A}	1.39 ± 0.62^{B}	0.008**
Loin muscle area (LMA) (cm2)	86.82 ± 25.44	74.26 ± 26.62	77.25 ± 15.16	0.083
Marbling score (MS) (1–5)	2.24 ± 0.83	2.00 ± 0.69	2.50 ± 1.20	0.277
Water holding capacity (WHC) (%)	0.22 ± 0.04	0.21 ± 0.03	0.22 ± 0.03	0.123
Meat tenderness (MT) (kg)	4.41 ± 0.53	4.28 ± 0.72	3.98 ± 0.28	0.098

Data with a different letters (A and B) within the same line are different significantly at $P < 0.01$, respectively; ** Effect was significant at $P < 0.01$.

Table 6. Association of 3514 A>T SNP genotypes with carcass and meat quality traits at bovine ATGL gene.

Trait	AA (Mean ± SD)	AT (Mean ± SD)	TT (Mean ± SD)	P-value
Live weight (LW) (kg)	503.14 ± 63.12	504.28 ± 52.69	500.75 ± 64.43	0.988
Carcass weight (CW) (kg)	265.96 ± 37.73	264.56 ± 35.42	272.80 ± 26.90	0.844
Dressing percentage (DP) (%)	0.53 ± 0.02^{a}	0.52 ± 0.02^{a}	0.55 ± 0.02^{b}	0.032*
Carcass length (CL) (cm)	146.50 ± 6.63	144.47 ± 8.14	148.25 ± 8.89	0.284
Carcass chest depth (CCD) (cm)	28.45 ± 3.08^{a}	29.33 ± 3.12^{ab}	31.75 ± 1.58^{b}	0.013*
Hind leg length (HLL) (cm)	72.79 ± 3.54	73.32 ± 3.68	73.63 ± 5.01	0.719
Backfat thickness (BFT) (cm)	1.05 ± 0.37	1.08 ± 0.35	0.89 ± 0.12	0.370
Loin muscle area (LMA) (cm2)	81.67 ± 28.01	83.08 ± 20.32	90.57 ± 29.69	0.656
Marbling score (MS) (1–5)	2.17 ± 0.80^{a}	2.06 ± 0.86^{ab}	3.00 ± 0.76^{b}	0.014*
Water holding capacity (WHC) (%)	0.22 ± 0.04	0.22 ± 0.04	0.24 ± 0.39	0.181
Meat tenderness (MT) (kg)	4.38 ± 0.65	4.33 ± 0.51	4.19 ± 0.33	0.696

Data with a different letters (a and b) within the same line are different significantly at $P < 0.05$, respectively; * Effect was significant at $P < 0.05$.

Akiyama et al., (2007) reported a 'CTCC' duplication at 475–478nt which resulted in frame shift of 178Ter in human *ATGL* gene, and subsequently a truncated peptide was translated. Both studies showed that the lipase truncated in its C-terminal domain lacks biological activity (Akiyama et al., 2007). In pigs, the *ATGL* gene expressed mainly in white adipose and muscle tissue and played a role in catecholamine-induced lipolysis in adipose tissue (Deiuliis et al., 2008). In our study, the data revealed that the genotypes CC and TT were present at low frequencies, accordingly, the mutant allele C and T had lower frequency when compared with the wild allele G and A in all the experimental populations. Comparisons of economic traits among individuals with different genotypes, individuals with genotype CC and TT had some superior carcass and meat quality traits, which indicate that allele C and T might be the beneficial alleles for some economic traits of QC cattle. These results could be explained that the two SNPs might be relevant to bovine carcass and meat quality traits. Both mutations induced changes in the amino acid sequence of ATGL protein, which may further affect the secondary structure and/or expression of the mRNA, protein structure and/or protein function during the progress of evolution (Komar, 2007; Shastry, 2009).

In conclusion, we first indentified two SNPs in the bovine *ATGL* gene. Genotyping and association analysis demonstrated that the two SNPs were significantly associated with carcass and meat quality traits in bovine. Therefore, in the future, it will be necessary to use larger population to investigate whether the two SNPs of *ATGL* gene play roles in those traits, and the two SNPs may be used for marker-assisted selection (MAS) to gain the genetic improvement of native Chinese bovine carcass

and meat quality traits.

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