Molecular characterization and association analysis of porcine adipose triglyceride lipase (*PNPLA2*) gene

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Abstract The adipose triglyceride lipase (PNPLA2, also known as ATGL) is a novel triacylglycerol (TG) lipase which specifically removes the first fatty acid from the triglyceride molecule generating free fatty acid and diglyceride (DG) in mammalian cells. Here we describe the molecular characterization of the porcine *ATGL* gene. The full-length cDNA sequence contains a 1,461 bp open reading frame encoding a protein of 486 amino acids with a calculated molecular mass of 53.2 kDa and an isoelectric point of 7.90. The porcine ATGL protein shares high identity with other mammalian ATGL. The *ATGL* gene contains 9 coding exons, spans approximately 6 kb. The porcine *ATGL* mRNA was expressed predominantly in backfat, mildly in muscle, small intestine and heart, and almost absent in liver, spleen, lung, stomach, kidney and ovary. Statistical analysis showed the *ATGL* gene polymorphism (G/A^{392}) was different between Chinese indigenous and introduced commercial western pig breeds, and was highly associated with almost all the fat deposition and carcass traits, including subcutaneous fat thickness, viscera adipose tissue, lean percentage, loin eye traits and even rib numbers.

Keywords ATGL · Pig · Characterization · Real-time PCR · Genomic walking · Association analysis

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Introduction

It has been believed that there was only one rate-limiting enzyme, hormone-sensitive lipase (HSL), for triglyceride metabolism in mammalian adipose tissue. However, this widely held views were changed after the phenotype that the HSL-knockout mice were not obese and still had accumulated diglycerides in adipocytes [1], which indicated that, even in the absence of HSL, there are unidentified lipases efficiently degraded triglycerides to diglycerides. In 2004, a novel triacylglycerol lipase, adipose triglyceride lipase (ATGL) [2], with the official name of patatin-like phospholipase domain containing 2 (PNPLA2), and alternative names of ζ-isoform of a calcium-independent phospholipase A2 (iPLA2ζ) [3], des-nutrin [4], transport-secretion protein 2.2 (TTS2.2) and pigment epithelium-derived factor-receptor (PEDF-R) [5], was identified and characterized simultaneously by several laboratories. It was described to predominantly perform the initial step in triglyceride hydrolysis [2] and identified as an important TG hydrolase in lipid droplets/adiposome turnover in mammalian cells, including lipid-storage cells and non-adipocyte cells [6]. So Smirnova et al. [6] suggested it should have the name of "adiposome triglyceride lipase" instead of "adipose triglyceride lipase". Moreover, it was

reported that ATGL and HSL are responsible for more than 95% of the TG hydrolase activity present in murine white adipose tissue [6]. Thus, lipid mobilization has been further modified to be a two-step affair, and it is believed that ATGL and HSL coordinately catabolize stored triglycerides in adiposome of mammals [2].

ATGL gene has been mapped in 11p15.5 on human chromosome, 7F5 on mouse chromosome and 1q41 on rat chromosome, respectively (http://www.informatics.jax.org/searches/homology_report.cgi?_Marker_key=50833). As for porcine ATGL gene, we have assigned it to porcine chromosome 2p17 (SSC2pter) by radiation hybrid mapping in the corresponding region [7].

As a nature model of obesity, pigs share similarity with human both in metabolism and physiology. Comparative analysis of obesity-related genes in the pig is not only important for development of marker-assisted selection on growth and fat deposition traits in the pig but also provides for an understanding of their genetic roles in the development of human obesity. There have been more and more genes proved to be important for pig fat deposition traits, such as FoxO1 [8], WNT10B [9], MAT2β [10], LCAT [11], APOM [12], Lpin1 [13] and so on.

In this study, we selected porcine ATGL as a functional candidate gene for fat deposition traits in pigs, and presented the expression profiles and molecular organization of porcine ATGL gene including cDNA and genome structures, single nucleotide polymorphisms and association with important economic traits. The results will provide a molecular foundation for further functional studies and applications.

Materials and methods

Full-length cDNA isolation

Primers 1F and 1R (Online Resource 1) designed from available ESTs in porcine EST-others database were used to amplify the whole coding cDNA sequence of porcine ATGL gene. PCR reactions were performed in a volume of 25 μl reaction volume containing 25 ng porcine cDNA, 1× GC buffer I (Takara, Japan), 0.2 μM each primer, 0.5 mM of each dNTP and 0.25U LA Taq DNA polymerase (Takara, Japan). The thermal cycling program consisted of 1 min at 94°C, followed by 9 cycles of 30 s at 94°C, 30 s at 68–60°C (touchdown PCR, annealing temperature was decreased 1°C after each cycle), and 90 s at 72°C. This touchdown step was followed by 26 cycles of 30 s at 95°C, 30 s at 60°C, and 90 s at 72°C. Gene specific primers (5'P1/2, 3'P1/2, Online Resource 1) according to the obtained cDNA sequence were used to amplify the full-length cDNA sequence of porcine ATGL from cDNA

library of backfat tissue of a 3-month-old Meishan pig following the instructions of TaKaRa 5'Full-RACE Kit and 3'Full-RACE Kit (TaKaRa, Japan).

Full-length genomic DNA sequence isolation

Primers 1F and 1R were used to amplify the genomic DNA sequence containing the whole coding region, while gene specific primers (3GSP1/2, 5GSP1/2; Online Resource 1) were used to obtain the 3'-flanking and 5'-flanking region according to the instructions of Universal Genome Walker™ Kit (Clontech Laboratories, Palo Alto, CA). All the major PCR products were purified, subjected to cloning (pGEM T Vector System I, Promega, USA) and sequencing.

Quantitative real-time PCR

Samples of heart, liver, spleen, lung, kidney, stomach, small intestine, ovary, longissimus dorsi muscle and backfat tissues were collected from a 4-month-old female Large White pig, and a real-time quantitative RT-PCR method with SYBR® Green PCR Master Mix (Toyobo, Japan) was carried out using an iCycler iQ system (Bio-Rad, USA). 18S rRNA was used as an internal control. Both of ATGL and 18S transcripts were amplified in 2 separate PCR reactions with specific primers (ATGL-qF/R, 18S-F/R; Online Resource 1) and all samples were run in quadruplicate. Each reaction mixture (25 μl) contained 5 ng cDNA, 800 nM forward and reverse primers, and 1× SYBR Green PCR Master Mix. SYBR Green Assay program for thermal cycling was as follows: 1 min at 95°C for one cycle and 40 cycles at 95°C for 15 s, 60°C for 15 s, followed by 25 s at 72°C. The expression level of ATGL was normalized to the expression level of 18S in each sample. This relative value was further normalized to the relative expression of ATGL in the pooled sample.

Polymorphism screening and association analysis

In order to identify polymorphisms in the porcine ATGL gene, overlapping PCR fragments were amplified from individual genomic DNA of three pig breeds.Two typical lean-type western breeds Large White (n = 30) and Landrace (n = 40) are chosen, as well as a typical Chinese indigenous breed Meishan (n = 47). The nucleotide sequences of each animal were compared to find out polymorphisms in porcine ATGL gene. Primer mutagenesis and PCR–RFLP were performed to genotype the polymorphic sites.

The association analysis was performed in 236 pigs of the "Large White × Meishan" F2 population. The records of fat deposition and carcass traits were measured according

to the method of Xiong and Deng [14], including skin percentage (SP), bone percentage (BP), fat meat percentage (FMP), lean meat percentage (LMP), ratio of lean to fat (RLF), shoulder fat thickness (SFT), 6–7th rib fat thickness (RFT), thorax-waist fat thickness (TFT), buttock fat thickness (BFT), average backfat thickness (ABT), leaf fat weight (LFW), caul fat weight (CFW), internal fat rate (IFR), loin eye height (LEH), loin eye area (LEA), carcass length (CL) and rib number (RN). Statistical analyses were performed using the SAS mixed model procedure (SAS procedure MIXED; SAS Institute. Cary, NC, USA), with a model that included sex, genotype and batch as fixed effects, sire and litter as random effects, and date or weight at slaughter as a covariate. Both additive and dominance effects were estimated using the REG procedure. Significant level was set to be 0.05.

Results

Characterization of porcine *ATGL* cDNA

A 2,076 bp full-length porcine *ATGL* cDNA (GenBank accession no. EF648448; Online Resource 2) was identified to has a 1,461 bp open reading frame (GenBank accession no. EF567018) encoding a protein of 486 amino acids with a molecular mass of 53.2 kDa and an isoelectric point of 7.90; flanked by a 144 bp-long 5′UTR and 594 bp of 3′UTR which contains a polyadenylation signal AATAAA and a poly(A) tail.

Predicted porcine ATGL shares high homology with other vertebrate proteins: 87% identity with cow (NP_001039470.1), dog (XP_854164.1) and mouse (NP_080078.1), 85% with rat (XP_341961.1), 83% with human (NP_065109.1) and 63% with chicken (XP_428618.1). Phylogenetic tree constructed using clustalW method of DNASTAR Megalign (DNASTAR Inc., Madison, WI) revealed that porcine ATGL has the closest genetic relationship with cow, and closer with mouse and rat than that of human.

Like other ATGL proteins, porcine ATGL is predicted to contain an N-terminal patatin-like domain (PFAM accession PF01734; amino acids 10–179; Online Resource 2) characteristic of a large family of storage proteins and lipid acyl hydrolases found in eukaryotes and bacteria ("conserved domains" blast in NCBI). This region also contains a predicted 'esterase of the α/β hydrolase fold' domain and a catalytic dyad (Gly-X-Ser-X-Gly and Asp-X-Gly/Ala) consensus sequence (Online Resource 2). Active residues of porcine ATGL correspond to Ser[47] and Asp[166]. The C-terminal region contains a proline-rich region (amino acids 460–485) (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Potential protein kinase C phosphorylation sites were found at nucleotide (nt) positions 93, 111, 215, and 354; casein kinase II phosphorylation sites at 68, 122, 300 and 433; N-myristoylation sites at 24, 45, 59, 115, 168, 422 and 451; N-glycosylation sites at 9 and 209 and one prokaryotic membrane lipoprotein lipid attachment site at 18 (PROSITE, PC gene) [15]. As shown in Online Resource 3, TopPred [16] predicted five transmembrane domains (TM), among which it was predicted to have an extracellular region between TM3 and TM4 and two intracellular regions of TM2–TM3 and TM4–TM5, which was almost at the same positions with the results of TMpred [17]. PSORT II (http://psort.nibb.ac.jp/form.html) predicted a plasma membrane protein and no N-terminal signal peptide.

Genomic structure of porcine *ATGL* gene

A length of 4,404 bp genomic sequence of porcine *ATGL* including the coding region was obtained with primers 1F/R. By genomic walking, a 1,982 bp 3′-flanking sequence and a 1,250 bp 5′-flanking sequence were obtained with specific primers 3GSP1/2 and 5GSP1/2, respectively. The 7 kb assembled sequence (GenBank accession no. EF567019) entirely contains whole genomic DNA sequence of porcine *ATGL* gene.

Comparison analysis with homogenes revealed that porcine *ATGL* gene consists of 9 coding exons (Table 1). All the intron–exon boundaries of various exons conform to the GT/AG rule for nucleotides immediately flanking exon borders [18]. As shown in Online Resource 4, the upstream 1.1 kb of the sequence of porcine *ATGL* gene is predicted to contain a promoter region (−205 to −155 bp from the start of translation) by NNPP on website (http://www.fruitfly.org/seq_tools/promoter.html), as well as potential cap, TATA, CAAT and GATA motifs on MotifFinder website (http://motif.genome.jp/motif-bin/Srch_Motif_Lib) and CREBP1, MyoD and C/EBP binding sites on SignalScan website (http://bimas.dcrt.nih.gov/molbio/signal/). The 3′-flanking sequence analysis shows a typical polyadenylation signals (AATAAA) downstream of the termination codon, which is consistent with the cDNA sequence obtained.

mRNA expression profile

Real-time RT-PCR was performed and revealed the mRNA expression of porcine *ATGL* gene in all of the selected organs. The mean threshold cycles for *ATGL* and 18S were 30.2 and 18.4 with standard deviations of 0.53 and 0.16, respectively. As shown in Fig. 1, porcine ATGL displayed a significantly high expression in backfat, mild in muscle, small intestine and heart, and almost absent in liver, spleen, lung, stomach, kidney and ovary. In comparison, the expression in backfat is about 15 times to muscle, 40 times

Table 1 Genomic organization of porcine *ATGL* gene

Exon	Exon size (bp)	Intron size (bp)	3′ Splice acceptor	5′ Splice donor
1	331	1823	None (5′UTR)	GTCTGCCTGGgtgag
2	234	89	tccagGTGATACTGG	CTCATCCAGGgtggg
3	65	320	cgcagCCAACGTCTG	CCAGGGCGAGgtaag
4	210	191	cacagCGCTACGTGG	CGAGCCCCTGgtgag
5	61	109	ctcagGTGCTTCGAG	CGGCGGAACGgtgcg
6	177	79	cccagGCCTCCTGAA	CTCAATGACGgtggg
7	133	170	cacagCCCTGCTGGA	TCACCGTCCGgtgcg
8	123	87	cccagCTTGCTGGAG	TGCCCTCCAGgtgag
9	723	None	cacagGCTGACGGAG	AATAAACGCCACAGCCCAG[a]

Coding sequences are shown in *uppercase letters* and noncoding regions in *lower letters*

[a] The 19 bases directly upstream of the poly(A) tail are indicated; the size of the 3′UTR excluding the poly(A) tail is 452 bp
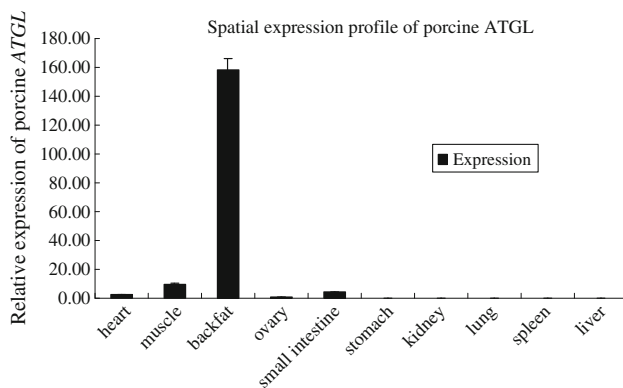

Fig. 1 Spatial expression profile of porcine *ATGL* mRNA in different tissues by real-time RT-PCR using an iCycler iQ system. The *horizontal axis* and *vertical axis* indicate different tissues and relative expression level of porcine *ATGL* mRNA. The expression level of porcine *ATGL* mRNA was normalized to 18s rRNA and measured with $2^{(-\Delta\Delta Ct)}$ value. Results are averaged from three independent replicates

to small intestine and 50 times to heart. The expression in other tissues can hardly be detected.

Polymorphism detection and association analysis

Eleven potential polymorphic sites were detected in porcine *ATGL* gene using direct sequencing by PCR after sequence analysis. Among these potential SNPs, a G/A[392] substitution which causes the 131 amino acid change of arginine to histidine (CGC-CAC, R/H[131]) was identified to locate in the patatin domain that is conserved in the PNPLA2 family. Using the mutant primers SNP-F/R (Online Resource 1), a 240 bp PCR product was generated and then digested by enzyme *Bpu*1102I, and three banding types were observed in 8% nondenaturing polyacrylamide gels: genotype AA was present in one band (240 bp), genotype GG in two bands (218, 22 bp), and genotype AG in three bands (240,

Table 2 Genotype distribution and allele frequencies in three pure breeds

Breed	Number	Genotype			Allele frequency	
		AA	AG	GG	A	G
Large white	30	16	12	2	0.73	0.27
Landrace	40	28	8	4	0.80	0.20
Meishan	47	0	25	22	0.27	0.73

218, 22 bp). Results of statistical analysis of genotypes and allele frequency can be seen in Table 2. Analysis of this SNP revealed a much higher frequency of allele G in Chinese indigenous breed Meishan (73%) whereas allele A had a higher frequency in Large White (73%) and Landrace (80%).

Statistical analysis showed the polymorphism was prominently associated with fat deposition traits and carcass traits, such as FMP ($P = 0.0034$), LMP ($P = 0.0006$), RFT ($P = 0.0012$), TFT ($P = 0.0083$), BFT ($P = 0.0283$), ABT ($P = 0.0080$), LEH ($P = 0.0111$), LEA ($P = 0.0052$), RN ($P = 0.0414$) and IFR ($P = 0.0036$). As shown in Table 3, there were highly significant or significant differences between traits of the AA genotypes and those of the GG genotypes: FMP ($P = 0.0008$), LMP ($P = 0.0001$), RLF ($P = 0.0024$), RFT ($P = 0.0003$), TFT ($P = 0.0039$), BFT ($P = 0.0089$), ABT ($P = 0.0044$), LFW ($P = 0.0062$), CFW ($P = 0.0144$), LEH ($P = 0.0028$), LEA ($P = 0.0016$), RN ($P = 0.0142$) and IFR ($P = 0.0009$). Significant or highly significant differences were also observed between traits of AG genotypes and those of GG genotypes in most of the fat deposition traits. Furthermore, this locus seemed to be significantly additive in action and allele A was associated with decrease of fat deposition. Pigs with AA genotype had less FMP (-2.32%), RFT (-0.31 cm), TFT (-0.26 cm), BFT (-0.31), ABT (0.24), LFW (-0.10 kg), CFW (-0.12 kg) and IFR (-0.38%), and

Table 3 Association between porcine *ATGL Bpu*1102I PCR–RFLP and fat deposition and carcass traits

Traits[1]	Genotype (Lsmean ± SE)[2]			Effect (mean ± SE)	
	AA (n = 108)	AG (n = 106)	GG (n = 22)	Additive	Dominance
SP (%)	9.66 ± 0.25	10.25 ± 0.26	10.54 ± 0.56	0.44 ± 0.31	0.15 ± 0.41
BP (%)	13.45 ± 0.28	13.26 ± 0.29	12.15 ± 0.62	−0.65 ± 0.34	0.46 ± 0.46
FMP (%)	24.01 ± 0.56[A,3]	24.64 ± 0.56[A]	28.65 ± 1.22[B]	2.32 ± 0.67**	−1.69 ± 0.90
LMP (%)	52.86 ± 0.44[A]	51.84 ± 0.45[A]	48.65 ± 0.97[B]	−2.11 ± 0.53**	1.08 ± 0.72
RLF	2.28 ± 0.07[A]	2.18 ± 0.07[a]	1.75 ± 0.15[Bb]	−0.26 ± 0.08**	0.17 ± 0.11
SFT (cm)	3.73 ± 0.10	4.02 ± 0.11	3.91 ± 0.22	0.09 ± 0.12	0.20 ± 0.16
RFT (cm)	2.82 ± 0.07[A]	2.97 ± 0.07[A]	3.44 ± 0.15[B]	0.31 ± 0.08**	−0.16 ± 0.11
TFT (cm)	2.09 ± 0.07[A]	2.30 ± 0.07[AB]	2.61 ± 0.16[B]	0.26 ± 0.09**	−0.05 ± 0.12
BFT (cm)	1.97 ± 0.10[A]	2.15 ± 0.10[AB]	2.59 ± 0.21[B]	0.31 ± 0.11**	−0.13 ± 0.15
ABT (cm)	2.65 ± 0.07[Aa]	2.86 ± 0.07[b]	3.14 ± 0.15[B]	0.24 ± 0.08**	−0.03 ± 0.11
LFW (kg)	0.69 ± 0.03[A]	0.75 ± 0.03[AB]	0.89 ± 0.07[B]	0.10 ± 0.03**	−0.04 ± 0.04
CFW (kg)	1.40 ± 0.04[a]	1.42 ± 0.04[a]	1.65 ± 0.09[b]	0.12 ± 0.04*	−0.11 ± 0.06
LEH (cm)	8.58 ± 0.10[A]	8.47 ± 0.10[a]	7.82 ± 0.22[Bb]	−0.38 ± 0.12*	0.26 ± 0.16
LEA (cm[2])	29.08 ± 0.60[A]	27.69 ± 0.61[a]	24.45 ± 1.31[Bb]	−2.32 ± 0.71**	0.92 ± 0.95
CL (cm)	91.18 ± 0.56	89.98 ± 0.57	89.40 ± 1.24	−0.89 ± 0.68	−0.31 ± 0.91
RN	14.58 ± 0.09[ab]	14.77 ± 0.09[a]	14.21 ± 0.21[b]	−0.19 ± 0.11	0.37 ± 0.15*
IFR (%)	3.32 ± 0.09[A]	3.41 ± 0.09[A]	4.09 ± 0.20[B]	0.38 ± 0.11**	−0.30 ± 0.15

[1] *SP* skin percentage, *BP* bone percentage, *FMP* fat meat percentage, *LMP* lean meat percentage, *RLF* ratio of lean to fat, *SFT* shoulder fat thickness, *RFT* 6–7 rib fat thickness, *TFT* thorax-waist fat thickness, *BFT* buttock fat thickness, *ABT* average backfat thickness, *LFW* leaf fat weight, *CFW* caul fat weight, *LEH* loin eye height, *LEA* loin eye area, *CL* carcass length, *RN* rib number, *IFR* internal fat rate

[2] Least square mean values (±SE)

[3] Different *letters* denoting significant difference between groups: a, b, * $P < 0.05$; A, B, ** $P < 0.01$

more LMP (2.11%), LEH (0.38 cm), LEA (2.32 cm[2]) and RLF (0.26) than pigs with GG genotype ($P < 0.05$).

Discussion

In present study, molecular characterization, expression profiles in tissues and association analysis of porcine *ATGL* were primarily discussed. The obtained whole cDNA sequence of porcine ATGL is consistent with Shan' study [19]. Consistent with other homogenes, porcine *ATGL* gene shares high similarity in gene structure, physical mapping and transcription expression level. The NH$_2$-terminal sequence of predicted porcine ATGL contains a patatin-like domain, characteristic of patatin, a potato protein with lipid acyl hydrolase, acyl transferase, and esterase activity [20, 21]. It also contains a predicted α/β hydrolase fold, a GXSXG serine hydrolase motif, and a Ser-Asp catalytic dyad, all features commonly found in known lipases [20]. The high level of homology may suggest the similar function of porcine ATGL in lipid metabolism and similar location in cytoplasm and lipid droplet surface. However, it needs to be tested experimentally. Furthermore, there is proline-rich domain in its C-terminal. Since proline favors turns or kinks for steric reasons, proline-rich activation

domains are likely to form an omega loop structure containing several reverse turns [22], and enable other residues to interact with the basic transcription machinery [23]. Banerji et al. [24] suggested that proline-rich motif may affect dimmer formation and be important for the proper conformation of the protein.

In this study, the real-time RT-PCR results indicate a specific expression of porcine *ATGL* mRNA, with highest in porcine backfat, in agreement with previous studies [2, 4, 19], but almost absent in liver, spleen, lung, stomach, kidney and ovary. In contract, it was reported to be expressed to a lesser degree in adipose tissue, kidney, heart, and muscle, and least but detectable in brain [19]. Besides, ATGL is also reported to be in the retina and speculated to produce extracellular bioactive lipids which can diffuse back into the cell as signaling molecules [5]. Data from significantly high expression of ATGL in adipose tissue may assign a central role to this enzyme for the catabolism of cellular fat stores.

Previous studies showed mutations in human *PNPLA2* gene were associated with degradation of lipid droplets and disorders related to fat deposition such as type II diabetes [25, 26]. As for obesity model pig, a substitution mutation of G/A^{392} (R/H^{131}) was first identified to be located in the highly conserved patatin domain. Notably, according to the

transmembrane regions predicted by TOPpred (Online Resource 3), R/H[131] variation locates just right at the cytoplasmic loops (64-140aa) adjacent to the TM3 (141-161aa). Since the variation in the functional region of patatin, it is probable that the change of R/H[131] could influence the spatial structure or activation level of ATGL. The allele frequency was obviously different with dominance for allele G in Chinese indigenous breeds, but dominance for allele A in introduced commercial western breeds. Interestingly, the comparison results show [131]H, which is the majority in Large White and Landrace pigs, is the conserved amino acid in many mammals such as human, mouse, rat, dog, cow and so on. The analysis showed that the polymorphism was significantly associated with almost all the fat deposition and carcass traits measured, including subcutaneous fat thickness, viscera adipose tissue, lean percentage, loin eye traits and rib numbers. Considering the lean nature of Large White and Landrace pigs and lard nature of Meishan pigs, it is probable that A^{392} (H^{131}) may be a preferable allele for triglycerides degradation, while the variation of G^{392} (R^{131}) may cause a change of ATGL function. Since the potential significance, the effect of this polymorphism should be examined in larger pig populations to estimate genetic predisposition, which might provide evidence to molecular genetic test and selection for pig breeding.

ATGL was reported to account for 60–70% of the neutral TG lipase activity in both wild-type and HSL null mice [2]. However, recent studies show that HSL has a higher capacity than ATGL to hydrolyse TG in vitro and *HSL*, but not *ATGL* gene expression shows a regulation according to obesity status and is associated with increased adipose tissue lipase activity [27]. From the results of knocking down of ATGL, Rydén et al. [28] concluded that both HSL and ATGL regulate basal lipolysis in human adipocytes, however, in contrast to findings in rodents, ATGL is of less importance than HSL in regulating catecholamine-induced lipolysis and is not influenced by obesity. Therefore, more studies on the biological role and regulation mechanism of ATGL among various species need to be carried on in future. Our present results provide basis of genetic structure of porcine *ATGL* gene and complement previous studies of ATGL in mammals, and further studies on the role of ATGL in pigs are underway.

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