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Molecular cloning, expression and polymorphism of the porcine apolipoprotein A5 gene in a Jinhua × Pietrain F_2 reference population

L. F. Zhang, X. L. Jiang, X. C. Hua, Y. P. Lu and N. Y. Xu[†]

College of Animal Science, Zhejiang University, Hangzhou, 310029, China

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As a newly described member of the apolipoprotein gene family, apolipoprotein A5 *(*APOA5*) has been suggested to play a key role in the triglyceride metabolism in both human and mice. The aim of this study was to identify the porcine (Sus scrofa)* APOA5 *gene, determine its mRNA and its mutations that are associated with lipid accumulation. The porcine* APOA5 *cDNA was amplified by reverse transcriptase polymerase chain reaction using the information of the mouse or other mammals. It had been determined that the open reading frame of the porcine* APOA5 *gene consists of 1092 bp, which encodes a predicted protein composed of 363 amino acids with a similarity to bovine (80.43%) and to human (78.47%). The expression analysis indicated that the porcine* APOA5 *gene was expressed in hypophysis, fat and liver. Twelve single nucleotide polymorphisms (SNPs), including 4 SNPs in the 5′ end, 1 SNP in second intron, 1 SNP in third exon and 6 SNPs in the 3′ end, were identified in the porcine* APOA5 *gene and genotyped on the Jinhua × Pietrain F_2 reference population, it had revealed that the SNP of C1834T was significantly associated with average backfat thickness and leaf fat weight (*$P < 0.01$ *and* $P < 0.05$*, respectively). In conclusion, this study has got basic information of the porcine* APOA5 *gene and provides evidence that the* APOA5 *gene could be a potential candidate gene for fat deposition.*

Keywords: porcine, *APOA5* gene, cloning, expression, polymorphism

Implications

The pig meat is the major consuming meat in China. Native pig breed exhibited excessive body fat deposition, which was one of the main problems in the pig industry. High content of fat is not only to decrease the market value of pig meat, but also to increase the raise costs. We try to find genetic markers that cause to whole body fat deposition. Apolipoprotein A5 gene is a major candidate gene for our targets. This study is potential for improving the pig meat quality.

Introduction

In China, a long-term goal was to decrease body fat in pig production, because most of native pig breeds had excessive body fat deposition, which can decrease feed conversion rate and the whole quality of pig meat. Recently, studies had shown many genes are associated with the adipose traits in pig, such as *FTO*, *CMYA1* or *leptin* gene (Silveira *et al*., 2008; Fontanesi *et al*., 2009; Xu *et al*., 2009).

The *apolipoprotein A5* (*APOA5*) gene, a new member of the apolipoprotein gene family, is rapidly being recognized as a key regulator of serum triglyceride concentration (van Dijk *et al*., 2004; Elosua *et al*., 2006). *APOA5* can enhance lipoprotein lipase (LPL) activity to accelerate the rate of LPL-mediated triglyceride hydrolysis to regulate triglyceride metabolism (Schaap *et al*., 2004; Rensen *et al*., 2005). It had been found that several haplotypes of *APOA5* gene were related with the significant changes in triglyceride level (Grallert *et al*., 2007; Nelbach *et al*., 2008). In rat, the percentage of body fat was significantly correlated with serum triglyceride (Paik and Yearick, 1978), and in the young females and male pigs, the triglyceride level was positively correlated with backfat thickness (Mersmann and MacNeil, 1985). Martin *et al.* (1985) also noted that the high backfat Ossabaw sows had higher serum triglyceride levels. In healthy men, high fat content was associated with elevated total serum triglyceride (Walton *et al*., 1995), and visceral abdominal fat was also positively associated with serum triglyceride level in Japanese (Taniguchi *et al*., 2002). The above studies indicated that fat deposition can be

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[†] E-mail: nyxu@zju.edu.cn

influenced by the concentration of circulating triglyceride and *APOA5* may play an important role in fat deposition. To date, several mammalian *APOA5* cDNA sequences, such as human, cattle, rat and mouse have already been cloned, but porcine *APOA5* is still unknown.

At present, tagging single nucleotide polymorphisms (tagSNPs) are selected in genes to represent other co-related SNPs in linkage disequilibrium (LD) with the tagSNPs because of its high efficiency. This method has widely used to analyze the association in family study between genotype and traits in human disease or animal quantitative traits (Jiang *et al*., 2006; Tang *et al*., 2006; Hivert *et al*., 2009). Based on the important role in the triglyceride homeostasis, *APOA5* is considered as a potential candidate gene for fat deposition in our study. In order to identify the porcine *APOA5* gene and the associations between *APOA5* and fat deposition, we had firstly cloned and sequenced the whole gene, then detected the SNPs of *APOA5*, and investigated the effects of tag SNPs of *APOA5* with fat deposition in the Jinhua × Pietrain F_2 reference population lastly.

Material and methods

Animal and tissue collection, RNA extraction and cDNA synthesis

The tissue samples of kidney, hypophysis, heart, hypothalamus, spleen, small intestine, muscle, adipose and liver were derived from crossbred of Landrace sire × Yorkshire of 210-day old. Total RNA from the tissues was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of total RNA was measured at 260 nm using the NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of total RNA were reverse transcribed using random primers and the Improm-II reverse transcriptase (Promega, Madison, WI, USA), and finally the product was stored at −20°C.

RT-PCR

The porcine expressed sequence tag (EST) databases were searched by Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/) based on the sequences of cDNAs of human and rat *APOA5* gene (accession numbers: NM_052968 and NM_080576), then 9 porcine ESTs (accession numbers: BP443998, BP446485, DY418089, CJ000488, BP446061, BP446847, DB808886, CJ000039 and BP442824) were selected, which assembled into a contig, with which a set of primers was designed for PCR amplification and sequencing. The cycling conditions comprised denaturation at 94°C for 3 min, 40 cycles at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min, and last extension at 72°C for 10 min.

Rapid amplification of cDNA 3′ end

The 3′ end of cDNA was amplified with the 3′ Takara full RACE Kit (Otsu, Shiga, Japan) according to the manufacture's protocol. First-stand cDNA was synthesized using the adaptor primer, then two rounds of 3′ RACE-PCRs were performed using the amplification primer, 3′ RACE outer primer and gene specific outer primer for first round; 3′ RACE inner primer and gene specific inner primer for the nested PCR (Table 1). The PCR was performed using the following conditions: 32 cycles of (94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min) for first PCR; and 32 cycles of (94°C for 30 s, 60°C for 30 s and extension at 72°C for 1 min) for nested PCR.

Amplification and sequencing of genomic region

Genomic BLAST programs at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) and Sanger institute (http://www.sanger.ac.uk/DataSearch/blast.shtml/) were used for homology searches. Genomic DNA was extracted from blood using standard phenol protocol. All PCRs were performed by 36 cycles of 94°C for 30 s, annealing at 55°C to 68°C for 30 s and extension at 72°C for 1 min, and last extension at 72°C for 10 min. All products were ligated into the pGEM-T easy vector system (Takara, Japan) and then transformed into competent *E. coli* DH5α cells. Plasmid DNA was purified and sequenced on ABI 3730 sequencer (ABI, Foster City, CA, USA).

Sequence analysis

The open reading frame (ORF) was found using the DNAStar software, and the nucleotide sequences were translated to the amino acids by the DNAMAN program. The amino acid sequences of APOA5 were downloaded from Genbank (*Homo sapiens*: NP_443200; *Bos Taurus*: NP_001076961), and then the BLAST analysis was made by the DNAMAN program.

Reference population and phenotypic traits

Jinhua × Pietrain resource population was developed from purebred Pietrain sires and Jinhua dams, including 4 F_1 boars and 24 F_1 sows and ~250 F_2 progeny. The Jinhua pig has high backfat thickness, while the Pietrain has a heavy muscle, which leads to low backfat thickness. The average backfat thickness (ABF) was measured on the left carcass at four locations (shoulder, sixth and seventh rib, last rib and gluteus medius). The leaf fat (LF) weight on the left side of each carcass was weighted by electronic scale. On the day before slaughter, the animals were weighed, with an average weight of 85 kg (70 to 100 kg). The average values of ABF and LF in Jinhua pig are 3.82 cm and 1.21 kg, respectively, and Pietrain pig had 1.83 cm of ABF and 0.35 kg of LF.

Polymorphism detection and genotyping assay development

All primers were listed in Table 1. Approximately 50 ng of genomic DNA were amplified in a final volume of 20 μl that contained 12.5 ng of each primer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U of Taq polymerases. The PCR conditions were carried out as follows: 94°C for 3 min, 36 cycles at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 50 s, and last extension at 72°C for 10 min. Then the PCR products of primers 3 to11 were sequenced for SNP discovery using ABI 3730 sequencer (ABI, USA) with a standard protocol. The direct sequencing approach with the products of

Table 1 *Primer sets designed for porcine* APOA5 *gene*

Set	Position	Sense/antisense	Function	PCR (T_m)	Size (bp)
1	CDs	F: 5′-ATGGCAAGCGTGGCTGTAGTTCTG-3′ R: 5′-TTAGGGCTCCCCCAGATGGTCGAG-3′	cDNA cloning and Tissue expression	60	1092
2	GSP1	R: 5′-AGAGAAGGGAAGAAGGAAGAAACT-3′	3′RACE	55	309
	GSP2	R: 5′-ATATCCCTGTTGGCCGATGCTGGT-3′		60	248
3	5′region	F: 5′-ATCCAGGATACATTTTGAAGCAA-3′ R: 5′-GCTGGAAGTTGATAGGATGAAGAT-3′	Sequencing	62	240
4	5′region	F: 5′-TGCCTCTCATTATGTCATCTTCAT-3′ F: 5′-CTGGGAAAACTGAGGTATCCTG-3′	Sequencing and SNP	63	709
5	5′region	F: 5′-GTCTGGGCAGTTGAGTAAGAGC-3′ R: 5′-ATGGAATGTGGACCTCAAACTATT-3′	Sequencing and SNP	65	637
6	Exon1,2 and intron1	F: 5′-TGGGAACTATGAACTAAGGACCTC-3′ R: 5′-GAGGTTGAGGAGTTGGGTAGTG-3′	Sequencing and SNP	65	796
7	Intron2	F: 5′-GCAAGTCTCCTGAGAGATGTCC-3′ R: 5′-CAGGAACTTGTCCATATTGTTGAG-3′	Sequencing	66	892
8	Exon3	F: 5′-AAGGCCCTCACTCTTGGTTC-3′ R: 5′-GCTGGTCCAAGTTTTTCTGAAT-3′	Sequencing and SNP	65	691
9	Exon3 and 3′region	F: 5′-ATTGGGCACCACGTGCAGGAG-3′ R: 5′-TGGCAACAGGCTTGCACCAGGT-3′	Sequencing and SNP	63	825
10	3′region	F: 5′-ATTTGTGGGAAGACATCAACTACA-3′ R: 5′-TATATAAGGAAATCCAGGCTCCAG-3′	Sequencing	64	670
11	3′region	F: 5′-AAACAAATGGTATGGGTTGTGA-3′ R: 5′-CCCCTGTGGCATGTAGAAGT-3′	Sequencing	60	196
12	Intron2	Forward inner primer (T allele): 5′-TAGTTCTGCCCGCTATCCTGGCCCGCT-3′ Reverse inner primer (C allele): 5′-GCTGCCTGCAGAGAGTCCTAAACAGCCAAG-3′ Forward outer primer: 5′-TTTCAACCACCCAGGCAGGGAAAGGCTT-3′ Reverse outer primer: 5′-TCTGTGCAAGAAAAGCACGGAGGCACCT-3′	ARMS-PCR for SNP	69	203 (T allele) 281 (C allele) 427 (from two outer primers)
13	Exon3	Forward inner primer (C allele): 5′-TGCAGATGCTCTCGCACAAGCTCACGGTC-3′ Reverse inner primer (G allele): 5′-AATGTGCGCGTGCAGGGCCTTGGCCATC-3′ Forward outer primer: 5′-CTGCGCGTGGTCGGAGAGGACACCAAGG-3′ Reverse outer primer: 5′-TGTCGTGGCGGAAAGCCTGGAGTCGCTG-3′	ARMS-PCR for SNP	60	199 (C allele) 288 (G allele) 430 (from two outer primers)
14	3′region	F: 5′-ATTTGTGGGAAGACATCAACTACA-3′ R: 5′-TATATAAGGAAATCCAGGCTCCAG-3′	RFLP for SNP	65	670
15	GAPDH (AF017079)	F: 5′-ATGGTGAAGGTCGGAGTGAAC-3′ R: 5′-TTACTCCTTGGAGGCCATGTG-3′	Control for tissue expression	60	1002

CD = coding sequence; GSP1 = gene specific primer1; F = forward; R = reverse; SNP = single nucleotide polymorphism; ARMS-PCR = amplification refractory mutation system; RFLP = restriction fragment length polymorphism; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

primers 4 to 6 and 8 to 9 were also used to detect the polymorphisms on all animals. Additionally, genotyping was performed with the products of primers 12 to 13 by ARMS-PCR (amplification refractory mutation system) (Ye *et al*., 2001), while a *BsrB*I site for restriction fragment length polymorphism in the products of primer 14.

Statistical analysis

The Hardy–Weinberg equilibrium of each mutation and LD among mutations in porcine *APOA5* gene was estimated using the HAPLOVIEW program (Barrett *et al*., 2005). Association analysis of the SNPs with the traits in reference population was performed using the MIXED model procedure of SAS v9.0 (SAS institute Inc., Cary, NC, USA) with the following model: $Y_{ijklmno} = \mu + bW_i + B_j + S_k + D_l + G_m + Li_n + e_{ijklmno}$, where $Y_{ijklmno}$ is the dependent variable (traits); μ is the general mean; W_i is live weight (kg) as a covariate; B_j the birth year; S_k the sex; D_l the age (days); G_m the genotype of SNPs; Li_n the random effect of litter and $e_{ijklmno}$ the random error. *P*-value < 0.05 or < 0.01 was considered statistically significant or highly statistically significant after Bonferroni correction.

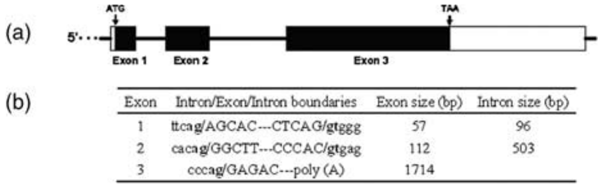
Results

Cloning of porcine APOA5 *gene*

On the basis of sequences of *APOA5* cDNA from other species, conserved regions of porcine *APOA5* gene were identified. Further analysis revealed that the cDNA of the porcine *APOA5* gene was composed of 1883 bp and were then submitted to the Genbank database (Accession numbers: FJ810861). The ORF was 1092 bp, with a predicted protein composed of 363 amino acid residues, which is 80.43% and 78.47% homologous to bovine and human APOA5, respectively. The information of genomic sequence released by the Porcine Genome Project, and the gene located in chromosome 9, CH242-243D19, CU582845. Genomic sequence of porcine *APOA5* was obtained by PCR. The exon-intron boundaries were further identified by the cDNA and genomic DNA alignment by DNAMAN software. The gene is distributed among three exons spanning of 2.482 kb of genome. All the exon-intron boundaries are consistent with the GT-AG splicing rule (Figure 1). Using the house-keeping gene GAPDH as internal control, RT-PCR analysis showed that the porcine *APOA5* gene was expressed in hypophysis, adipose and liver (Figure 2).

Single and multiple nucleotide polymorphisms

In the porcine *APOA5* gene, twelve SNPs were detected (Figure 3). The promoter region (spaning −1200 to +8 bp) harbors four SNPs, while the exons and introns contain two SNPs (G1295C and C400T). The remaining six SNPs are in the 3′ end region. No missense mutation occurs in the coding region. All mutations belonged to porcine genomic DNA contig (CU582845).



Exon	Intron/Exon/Intron boundaries	Exon size (bp)	Intron size (bp)
1	ttcag/AGCAC---CTCAG/gtggg	57	96
2	cacag/GGCTT---CCCAC/gtgag	112	503
3	cccag/GAGAC---poly (A)	1714	

Figure 1 (a) Schematic representation of the genomic structure of the porcine *APOA5* gene. Translated regions are given in black. (b) Exon/intron boundaries of the porcine *APOA5* gene. Intron sequences are shown in lowercase, and exon sequences are in uppercase. Both exon and intron sizes are indicated. The ag/gt consensus splice sequences are in boldface.

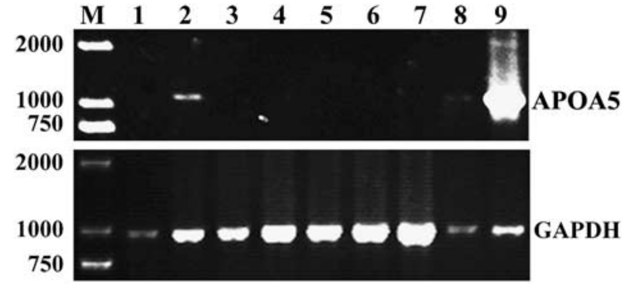


Figure 2 Tissue expression distribution of porcine *APOA5* gene. M, DL2000 markers; 1, kidney; 2, hypophysis; 3, heart; 4, hypothalamus; 5, spleen; 6, small intestine; 7, muscle; 8, adipose; 9, liver. *APOA5* = Apolipoprotein A5; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Haplotype analysis

The analysis of genotype data of all F_2 progeny indicated that two SNPs in the promoter region: G-769T and G-323A form two haplotypes GG and TA. No historical recombination status between these two SNPs was confirmed by HAPLOVIEW. In the 3′ end region, HAPLOVIEW also indicated that C1696T, T1697A, A1810G and T1940G have no historical recombination by forming two haplotypes of CTAT and TAGG. Therefore eight tagging SNPs, G-1013A, G-769T, G-458T, C400T, G1295C, A1810G, C1834T and G2107T, were used in the association analysis.

Association analysis of APOA5 *gene with ABF and LF*

The results of MIXED procedure revealed that the SNP of C1834T was significantly associated with ABF and LF in the population ($P = 0.0018$ and $P = 0.0213$, respectively) (Table 2). The CC animals had 0.306 cm of ABF less than the TT animals and 0.211 cm less than the CT heterozygote. Animal with GG genotypes had 0.09 kg of LF less than animals with TT. No any significance level was found to associate the other tag SNPs with ABF or LF.

Discussion

In this study, we described the identification and characterization of the full length of porcine *APOA5* cDNA and *APOA5* gene. The ortholog of porcine *APOA5* is related to its human and bovine counterparts based on cDNA and amino acid sequence comparisons. In human and rat, the expression of *APOA5* is mainly restricted to liver (van der Vliet *et al*., 2001). The porcine *APOA5* is weakly expressed in adipose and over expressed in liver.

A total of 12 mutations were detected in porcine *APOA5* gene region including a multiple nucleotide polymorphism in the promoter region. No any polymorphism association with ABF and LF were detected in the region of promoter, intron or coding regions. In the 3′ end region, the SNP of C1834T yielded strong association with ABF and LF ($P < 0.01$ and $P < 0.05$, respectively) (Table 2). In human, several SNPs within the *APOA5* locus (−1131T > C, −3A > G, S19W, IVS3 + 476G > A, 1259T > C and 1764C > T) have been identified, and their alleles are associated with triglyceride homeostasis in different populations (Nabika *et al*., 2002; Lai *et al*., 2004; Klos *et al*., 2005; Moreno-Luna *et al*., 2007). At the same time, two major haplotypes (−1131T > C and c.56C > G) associated with higher triglyceride levels had tagged (Pennacchio *et al*., 2001; Lai *et al*., 2007). Yao *et al*. (2008) also detected 7 mutations (C-169T, C600T, T635C, C841G, C914T, C1142G and C1394T) in chicken *APOA5* gene. In our study, SNPs were detected in porcine are different from the results of other species. This was not surprising because we reported for the first time the SNPs of porcine association with adipose traits.

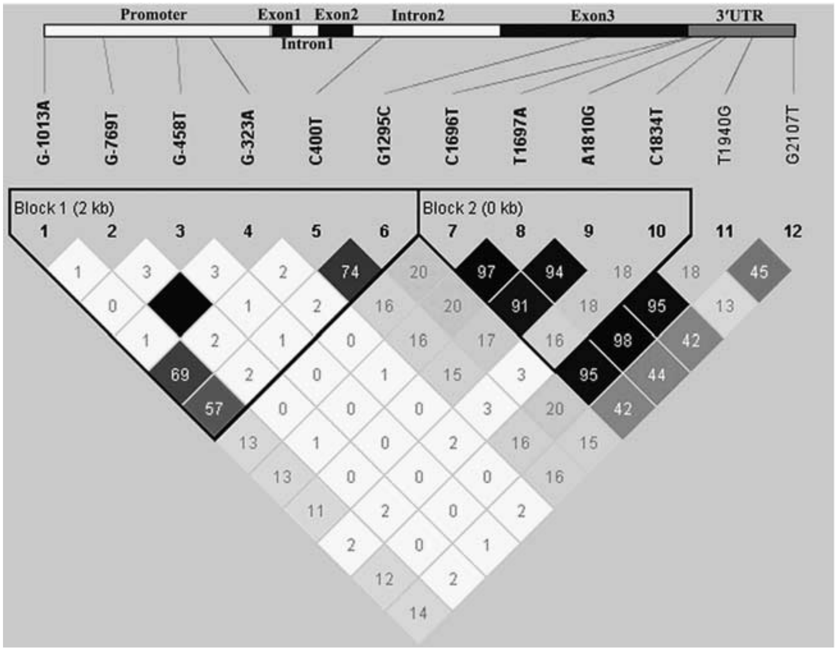


Figure 3 Genomic and haplotype analysis in the porcine *APOA5* gene. Pairwise linkage disquilibrium relationship for 12 mutations is noted based on r^2 measurements.

Table 2 *Associations of* APOA5 *gene polymorphism with ABF and LF*

Marker	Genotype	*n*	Carcass ABF thickness		Carcass LF weight	
			LSM ± s.e.	*P*	LSM ± s.e.	*P*
G-1013A	GG	175	3.224 ± 0.073^{a}	0.6152	0.839 ± 0.025^{a}	0.1257
	GA	58	3.190 ± 0.099^{a}		0.850 ± 0.035^{a}	
	AA	2	3.505 ± 0.346^{a}		1.103 ± 0.130^{a}	
G-769T	GG	166	3.190 ± 0.079^{a}	0.3679	0.839 ± 0.027^{a}	0.7725
	GT	69	3.266 ± 0.088^{a}		0.848 ± 0.031^{a}	
G-458T	GG	211	3.247 ± 0.076^{a}	0.1410	0.847 ± 0.024^{a}	0.3900
	GT	20	3.176 ± 0.148^{a}		0.817 ± 0.053^{a}	
	TT	4	2.706 ± 0.271^{a}		0.710 ± 0.103^{a}	
C400T	CC	161	3.225 ± 0.074^{a}	0.7768	0.838 ± 0.025^{a}	0.5226
	CT	74	3.202 ± 0.096^{a}		0.857 ± 0.034^{a}	
G1295C	GG	160	3.227 ± 0.075^{a}	0.6900	0.840 ± 0.025^{a}	0.7118
	GC	75	3.197 ± 0.094^{a}		0.850 ± 0.033^{a}	
A1810G	AA	63	3.172 ± 0.089^{a}	0.6271	0.811 ± 0.031^{a}	0.1769
	AG	126	3.236 ± 0.080^{a}		0.843 ± 0.027^{a}	
	GG	46	3.249 ± 0.094^{a}		0.877 ± 0.033^{a}	
C1834T	CC	85	3.057 ± 0.085^{Aa}	0.0018	0.793 ± 0.030^{a}	0.0213
	CT	113	3.268 ± 0.079^{ABb}		0.857 ± 0.027^{ab}	
	TT	37	3.363 ± 0.097^{Bb}		0.883 ± 0.035^{b}	
G2107T	GG	44	3.063 ± 0.096^{a}	0.0509	0.786 ± 0.034^{a}	0.0615
	TG	128	3.267 ± 0.078^{a}		0.858 ± 0.027^{a}	
	TT	63	3.265 ± 0.088^{a}		0.864 ± 0.031^{a}	

ABF = average backfat; LF = leaf fat; LSM = least significant mean; s.e. = standard error; *n* = number of animals genotyped.
Different superscript letters are significant differences (capital letters: $P < 0.01$; lowercase letters: $P < 0.05$).

APOA5 is located in the *ApoA1/C3/A4* gene cluster (van der Vliet *et al.*, 2001). Polymorphisms in this cluster have been linked to the human diseases in lipid metabolism (Mar *et al.*, 2004; Olivier *et al.*, 2004; Shanker *et al.*, 2008). Obviously the *APOA5* gene should be a strong candidate gene for the triglyceride metabolism. In *APOA5*-knockout mice, the triglycerides concentration increased fourfold (van der Vliet *et al.*, 2001), while the serum triglyceride concentrations decreased by 50% to 70% in transgenic mice with the human *APOA5* (Baroukh *et al.*, 2004). *APOA5* may enhance LPL-mediated triglyceride hydrolysis *in vitro* and modulate hepatic very low density lipoprotein-triglyceride synthesis to affect triglyceride levels (Fruchart-Najib *et al*., 2004; Schaap *et al*., 2004; Merkel *et al*., 2005). Additionally, *APOA5* has a unique association with cellular lipid droplets, which it may be involved in the storage or mobilization of intracellular lipids (Shu *et al*., 2007 and 2008). In chicken, the significant association between SNPs in T635C and abdominal fat weight in F_2 cross of White Plymouth Rock × Silkies were found (Yao *et al*., 2008). Our results confirmed the significant relationship of SNPs in *APOA5* gene and body fat deposition in porcine. Although the exact reasons for these finding are not clear, it speculated that *APOA5* gene may be a functional factor in fat deposition. Our research also provided a foundation for further investigation on function of *APOA5* gene.

In conclusion, we cloned the porcine *APOA5* gene using a comparative bioinformatics approach and developed a total of 12 genetic markers in this gene. Genotyping these markers on ~250 Jinhua × Pietrain F_2 reference population indicated that porcine *APOA5* gene is significantly associated with ABF and LF weight. We propose *APOA5* as a candidate gene for fat deposition, and further studies are needed to evaluate the effects of this gene in other pig breeds and investigate the regulating mechanism of *APOA5* in triglyceride metabolism.

Acknowledgments

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