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Effects of ATP Citrate Lyase and Adipose Differentiation-Related Protein Gene Polymorphisms on Adipose Deposition and Meat Quality Traits in Pigs

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Abstract: Adipose genes are potential candidates for meat quality. In the present study, two ESTs, coding for ATP-Citrate Lyase (ACL) and Adipose Differentiation-Related Protein (*ADRP*) gene were isolated via mRNA differential display technique. A C/T SNP in the 5'-flanking region of *ACL* gene and an insertion/deletion mutation in the fourth intron of *ADRP* gene were found, respectively. The selected pigs were genotyped at porcine ACL XhoI-RFLP and ADRPinsertion/deletion mutation. The results showed that the ACL genotypes presented a significant effect on leaf fat weight ($p<0.01$), caul fat weight ($p<0.05$), pH (LD) ($p<0.01$) and water holding capacity ($p<0.05$). Moreover, this site seemed to be significantly dominant in action ($p<0.01$ for leaf fat weight, caul fat weight and pH (LD); $p<0.05$ for water holding capacity) and allele B was associated with increase of both leaf and caul fat weights whereas with decrease of meat pH of Longissimus Dorsi and water holding capacity. The ADRP genotypes showed significance both on leaf fat weight and water moisture ($p<0.05$). The additive effects were significant for these traits while allele D increased the traits' phenotypic values. Based on this research, we proposed that both of two loci were potential makers for adipose deposition and meat quality traits.

Key words: ACL, ADRP, pigs, polymorphism, association analysis, China

INTRODUCTION

In pigs, the regulation of fat deposition is of major interest because desirable meat production is correlated to lean meat percentage and to favorable meat quality. Fat deposition is one of the important economic traits which show continuous variations and therefore, their underlying genetic nature is rather complex. With the development of animal genetics, candidate gene approach has become one of the commonly workable methods to identify both causative genes of these traits and the underlying causal mutations (Curi *et al*., 2006).

Adipose genes are potential candidates for production and meat quality. Hence interest is growing in studying the structural fat genes and their possible relationship with qualitative and quantitative characteristics of meat. Analyzing the differential genes expression has been proven to be essential and effective for the identification of novel candidate genes. Previously, there is evaluated the differential gene expression between F1 Meishan x Large White and Large White x Meishan hybrids and their parents by mRNA differential display technique. Among the differentially expression ESTs we recently isolated two representing *ACL* and *ADRP* gene, respectively. *ACL* gene was highly expressed in F1 hybrids.

In the present study, there is investigated the effects of ACL XhoI-RFLP and ADRP insertion/deletion polymorphism on fat and meat quality traits in the population derived from crossing Chinese Meishan and Large White pigs with the aim to identify DNA markers that could be used for porcine Marker Assisted Selection (MAS).

MATERIALS AND METHODS

Animals and traits' phenotypic values measurement: Two western commercial breeds, Large White and Landrace and two Chinese indigenous pigs, Meishan and Tongcheng were sampled from Jingpin pig station of

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Huazhong Agricultural University. Another four Chinese pig breeds, namely Hezuo, Bamei, Erhualian and Huainan which came from Gansu, Gansu, Jiangsu and Anhui province, respectively were also used in this study.

F2 generation (85 dams and 95 sires) of an intercross between Large White boars and Chinese Meishan sows was produced by mating 5 males to 16 females in the F1 generation in 2003. They were fed with same diets formulated according to age under a standardized feeding regimen and free access to water at Jingpin pig station of Huazhong agricultural University. The animals were slaughtered at the age of 6 months. Fat and meat quality traits phenotypic values including fat meat percentage, leaf fat weight, caul fat weight, average backfat thickness, meat pH of Longissimus Dorsi (pH(LD)), drip loss, water holding capacity, Meat Color Value of Longissimus Dorsi (MCV(LD)), Meat Marbling of Longissimus Dorsi (MM(LD)), intramuscular fat and water moisture were measured according to the method of Xiong and Deng (1999). Genomic DNA was prepared from blood samples using a standard phenol:chloroform extraction method.

Isolation of ACL and ADRP: Total RNA was isolated with TRIzol Reagent (Invotrigen, USA) from the porcine backfat at Thorax-Waist of six Large White and Meishan at four months old and mixed to RNA pools, respectively. cDNAs were synthesized using M-MLV reverse transcriptase and Oliog dT15 anchored primer (Promega, USA).

Differential display PCR and the non-denaturing polyacrylamide gel electrophoresis were employed by following the method described by Ren *et al*. (2005). The cDNA fragments which were differentially displayed in gel were re-amplified and sequenced. After that the obtained sequences were used to design primers for positive verification and followed by comparing these sequences with those available in Gen Bank using BLAST.

PCR amplification: According to the obtained sequences and the BLAST results, two gene-specific primers AD (forward: 5'-AGCTGCATCATCCGACTT-3'; reverse: 5'-GCCATTGCCAACACTTAC-3') and AC (forward: 5'-CGCCTTCCTAGCCCC-ACCT-3'; reverse: 5'-CGCCGCCTACCTTCCGGAG-3' (Ren *et al*., 2008) were designed to amplify ACL and ADRP, respectively. The reaction mixes comprised of over 50 ng porcine genomic DNA as template, 0.25 μM of each primer, 0.25 μM of each dNTP, 1×PCR buffer and 1U Taq DNA polymerase (Biostar Internation, Canada). The PCR amplifications were performed in 25 μL on a GeneAmp PCR system 9600 (Perkin Elmer, USA) with the following cycling parameters: denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C (the same for both ACLand ADRP) for 40 sec and extension at 72°C for 1 min. Finally, an additional extension for 10 min at 72°C was employed.

SNP identification and PCR-RFLP analysis: Using the genomic DNA of three Meishan and three Large White pigs as template, there is amplified *ACL* and *ADRP* gene by two primer pairs, AD and ACand then sequenced the amplicons, respectively to discover SNPs and other mutations.

The polymorphisms of ACL were analyzed by means of the PCR-RFLP technique with the following protocol: 6 μL of PCR products were digested with 5 U of Xho I (Fermentas, Lithuania) at 37°C overnight in a volume of 10 μL and the digested products were electrophoresed on 1.0% agarose gel and stained with ethidium bromide.

Association analysis: Association analyses were performed among the experimental populations that contained 180 Meishan x Large White F2 pigs. A General Linear Model (GLM) program of SAS version 8.1 software package (SAS Institute, USA) was performed to evaluate the associations between genotypes and production traits. Both additive and dominant effects were also estimated using REG procedure of SAS version 8.1 (SAS Institute, USA) where the additive effect was denoted as -1, 0 and 1 for genotype AA (CC), AB (CD) and BB (DD), respectively while the dominance effects were represented as 1, -1 and 1 for AA (CC), AB (CD) and BB (DD), respectively (Liu, 1998). The model of SAS program was as follows:

$$Y_{ijk} = \mu+G_i+S_j+B_k+e_{ijk}$$

Where:

μ = Represents the population mean
Y_{ijk} = Represents phenotypic value of the target trait
G_i = Represents the genotype effect
S_j = Represents the sex effect
B_k = Represents the boar effect
e_{ijk} = Represents random error effect for each observation

RESULTS

Isolation of ACL and ADRP: About >1000 ESTs were observed in differential display gels (Ren *et al*., 2005). Two ESTs, designated EST39 and EST40, demonstrated high-level expression in F1 hybrids compared with their parents by RT-PCR. Sequence analysis indicated that the cDNA fragments EST39 and EST40 were highly homologous to human *ACL* and porcine *ADRP* gene, respectively.

An 870 bp sequence in the 5'-flanking region of porcine *ACL* gene was obtained by genome walking

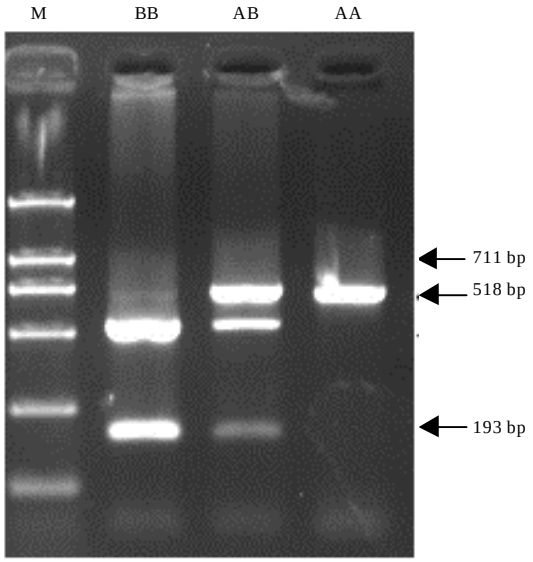

Fig. 1: The electrophoretic pattern of porcine ACL XhoI-RFLP. The genotypes are shown at the top of the lanes. M is Marker DL2000 (2000, 1000, 750, 500, 200 and 100 bp TaKaRa)

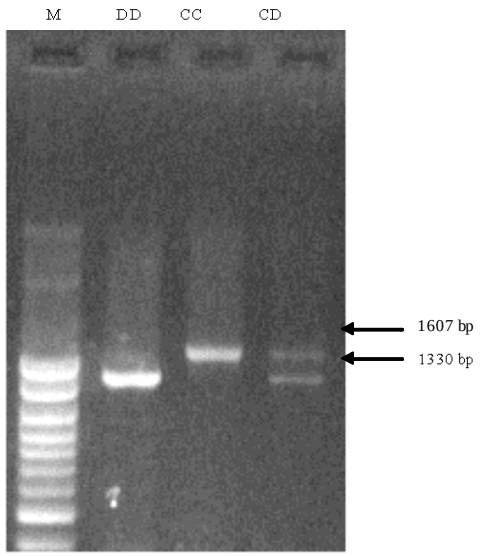

Fig. 2: Agarose gel electrophoresis showing three genotypes of ADRP insertion/deletion mutation. The genotypes are shown at the top of the lanes. M represents DNA marker whose maximum fragment has 1500 bp

based on Thermal Asymmetric Interlaced PCR in the previous study (GenBank Accession No. EU073663) (Ren *et al*., 2008) and 1607 bp fragment encompassing part of exon 4, exon 5 and complete intron 4 of porcine ADRP were obtained by PCR amplification (Gen Bank Accession No. AY621062).

Polymorphism detection of ACL and ADRP: The PCR products, 870 bp 5'-flanking sequence of ACL and 1607 bp sequence of ADRP, obtained from three different individuals representing two pig breeds (Large White and Meishan) were sequenced to search for potential polymorphisms.

In the 5'-flanking region of porcine ACL amplicon, 4 SNPs were found, C558A, T443C, A233C and C97T. The ACL C97T introduced an XhoI recognition site in the presence of T, resulting in the digestion of the 711 bp fragment, amplified by primer AC, into two 518 and 193 bp fragments, consequently forming three genotypes AA, AB and BB (Ren *et al*., 2008). The resulting electrophoretic patterns are shown in Fig. 1. In porcine ADRP amplicon, 3 SNPs and one insertion/deletion mutation (277 bp) were found. About 277 bp insertion/deletion mutation resulted in three genotypes, CC, CD and DD observed in Fig. 2.

Genotyping and association study: In order to investigate the possible relationships between carriers of different genotype and the trait values, the ACL XhoI PCR-RFLP and insertion/deletion mutation polymorphism of ADRP amplicon were genotyped in 8 purebred lines and Large White x Meishan F2 generation. Both genotype AA and allele A of ACL XhoI PCR-RFLP were dominant in all breeds whereas BB was only observed in Meishan pigs (Table 1). Genotype and allele frequency analysis of the insertion/deletion mutation polymorphism of ADRP in 308 unrelated animals revealed the allele C was dominant in all breeds except for Erhuanlian pigs (Table 2).

Table 1: Distribution of genotypic and allelic frequencies of ACL in the different pig populations

		Genotype number and frequency			Allele frequency	
Populations	No.	AA	AB	BB	A	B
Large white	58	45 (0.78)	13 (0.22)	0 (0.00)	0.89	0.11
Landrace	10	6 (0.60)	4 (0.40)	0 (0.00)	0.80	0.20
Meishan*	33	6 (0.18)	22 (0.67)	5 (0.15)	0.52	0.48
Hezuo	18	5 (0.28)	13 (0.72)	0 (0.00)	0.64	0.36
Bamei	39	31 (0.79)	8 (0.21)	0 (0.00)	0.90	0.10
Ehualian	17	11 (0.65)	6 (0.35)	0 (0.00)	0.82	0.18
Tongcheng	19	15 (0.79)	4 (0.21)	0 (0.00)	0.90	0.10
Huainan	40	32 (0.80)	8 (0.20)	0 (0.00)	0.90	0.10

The genotypic and allelic frequencies of ACL in Large White and Meishan pigs referred to Ren *et al.* (2008)

Table 2: Distribution of genotypic and allelic frequencies of *ADRP* in the different pig populations

		Genotype number and frequency			Allele frequency	
Populations	No.	AA	AB	BB	A	B
Large white	54	54 (1.00)	0 (0.00)	0 (0.00)	1.00	0.00
Landrace	14	12 (0.86)	0 (0.00)	2 (0.14)	0.86	0.14
Meishan	68	30 (0.44)	18 (0.27)	20 (0.29)	0.57	0.43
Hezuo	10	10 (1.00)	0 (0.00)	0 (0.00)	1.00	0.00
Bamei	24	17 (0.71)	5 (0.21)	2 (0.08)	0.81	0.19
Ehualian	15	3 (0.20)	4 (0.27)	8 (0.53)	0.33	0.67
Tongcheng	66	24 (0.36)	35 (0.53)	7 (0.11)	0.63	0.37
Huainan	57	28 (0.49)	20 (0.35)	9 (0.16)	0.67	0.33

Table 3: Association analysis of porcine ACL XhoI-RFLP genotype with fat and meat quality traits

	Genotype (μ±SE)			Genetic effect (μ±SE)	
Traits	AA (48)	CD (96)	BB (28)	Addictive	Dominance
Fat meat percentage (%)	0.255±0.006	0.239±0.004	0.249±0.008	0.003±0.005	-0.007±0.0030
Leaf fat weight (kg)	0.831±0.032^{A}	0.696±0.023^{Bb}	0.820±0.042^{a}	0.005±0.027	-0.065±0.0170**
Caul fat weight (kg)	1.471±0.038^{a}	1.355±0.027^{b}	1.459±0.051^{a}	0.007±0.032	-0.055±0.0210**
Average backfat thickness (mm)	2.870±0.080	2.766±0.056	2.889±0.106	-0.006±0.067	-0.054±0.0430
pH (LD)	6.325±0.018^{A}	6.335±0.013^{A}	6.412±0.025^{B}	-0.043±0.010	-0.0170±0.0100**
Drip loss (%)	7.009±0.259	6.649±0.183	6.162±0.345	0.423±0.217	0.031±0.1410
Water holding capacity (%)	90.498±0.344^{a}	90.980±0.242^{ab}	91.639±0.457^{b}	-0.570±0.288	-0.041±0.1871*
MCV (LD)	19.169±0.208	18.974±0.146	18.515±0.276	0.327±0.174	0.066± 0.1130
MM (LD)	3.553±0.025	3.568±0.017	3.568±0.033	-0.007±0.021	0.004±0.0130
Intramuscular fat (%)	3.750±0.068	3.751±0.048	3.759±0.091	-0.004±0.057	-0.002±0.0370
Water moisture (%)	73.710±0.110	73.702±0.078	73.731±0.146	-0.010±0.092	-0.009±0.0600

All the data in the table are least square means±standard error; Values in each line with different lower-case superscripts are significantly different at p<0.05, with capital superscripts different at p<0.01; Negative values of the additive effects denote a decrease of the trait value due to B allele; *p<0.05 and **p<0.01

Table 4: Association analysis of porcine *ADRP* insertion/deletion mutation polymorphisms with fat and meat quality traits

	Genotype (μ±SE)			Genetic effect (μ±SE)	
Traits	CC (106)	AB (29)	BB (29)	Addictive	Dominance
Fat meat percentage (%)	0.249±0.004	0.250±0.008	0.249±0.0080	-0.001±0.005	0.001±0.005
Leaf fat weight (kg)	0.742±.0226^{a}	0.721±0.043^{a}	0.843±0.0430^{b}	-0.050±0.024*	-0.036±0.025
Caul fat weight(kg)	1.423±0.029	1.366±0.055	1.400±0.0552	0.012±0.031	-0.023±0.032
Average backfat thickness (mm)	2.846±0.055	2.693±0.106	2.836±0.1060	0.005±0.060	-0.074±0.061
pH (LD)	6.359±0.013	6.342±0.024	6.321±0.0240	0.009±0.014	-0.014±0.014
Drip loss (%)	6.704±0.181	6.655±0.343	6.580±0.3430	0.062±0.195	0.006±0.198
Water holding capacity (%)	90.917±0.240	90.958±0.455	91.089±0.4560	-0.086±0.258	-0.022±0.261
MCV (LD)	18.889±0.140	18.993±0.265	18.943±0.2650	-0.027±0.150	0.039±0.152
MM (LD)	3.572±0.017	3.551±0.032	3.569±0.0320	0.002±0.018	-0.010±0.019
Intramuscular fat (%)	3.721±0.047	3.881±0.088	3.702±0.0880	0.010±0.050	0.085±0.051
Water moisture (%)	73.766±0.075^{ab}	73.485±0.142^{a}	73.782±0.1420^{b}	-0.008±0.049*	-0.144±0.081

All the data in the table are least square means±standard error; Values in each line with different lower-case superscripts are significantly different at p<0.05, with capital superscripts different at p<0.01; Negative values of the additive effects denote a decrease of the trait value due to B allele; *p<0.05 and **p<0.01

Within Large White x Meishan F2 population the genotype distributions of both *ACL* and *ADRP* gene were in Hardy-Weinberg equilibrium. The associations of tests for both genotypes and fat and meat quality traits were shown in Table 3 and 4. The ACL genotypes showed a significant effect on leaf fat weight (p<0.01), caul fat weight (p<0.05), pH (LD) (p<0.01) and water holding capacity (p<0.05). This site seemed to be significantly dominant in action (p<0.01 for leaf fat weight, caul fat weight and pH (LD); p<0.05 for water holding capacity) and allele B was associated with increase of both leaf and caul fat weights whereas with decrease of pH (LD) and water holding capacity. The ADRP genotypes showed significance both on leaf fat weight and water moisture (p<0.05). The additive effects were significant for these traits and allele D increased the traits phenotypic values.

DISCUSSION

ATP-citrate Lyase (ACL) catalyzes the critical reaction linking cellular glucose catabolism and lipogenesis, converting cytosolic citrate to acetyl-Coenzyme A (CoA). Acetyl-CoA is further converted to malonyl-CoA, the essential precursor for fatty acid biosynthesis (Kornacker and Ball, 1965). ACL is considered as one of the lipogenic enzymes like fatty acid synthase and acetyl CoA carboxylase. So, ACL could serve as a potential candidate gene for the traits of adipose deposition in porcine marker-assisted selection. In terms of the *de novo* lipogenesis state, changes in ACL activity are contributed to alterations in the rate of its biosynthesis (Akihiko *et al*., 1986) and correlate with modifications of mRNA concentration and transcription rate (Kim *et al*., 1992).

Several studies have founded that ACL expression in liver is regulated by SREBP-1 (Sato *et al*., 2000), sp1 (Moon *et al*., 2002) and NF-Y (Moon *et al*., 2000). These findings strongly suggest that ACL activity is regulated at the transcription level. In our previous study, researchers found a C/T mutation at position -97 bp upstream from the transcription start site. The transcriptional activity of promoter with allele C was significantly higher than that with allele T (p<0.01) (Ren *et al*., 2008). Thus, ACL mRNA will be more abundant in pigs with allele C than with allele T and more fat may be deposited in former pigs accordingly. It was consistent with the association analysis, which showed that the pigs with CC had more leaf and caul fat weights in the present study. Therefore, porcine ACL XhoI-RFLP is likely a useful marker for adipose deposition and meat quality traits. The *ADRP* gene was first cloned in mice (Jiang and Serrero, 1992).

ADRP is a ubiquitously expressed PAT family protein that plays important role as a fatty acid binding protein in

lipid droplet formation (Imamura *et al*., 2002). The protein has fatty acid-binding properties and stimulates fatty acid uptake in cells (Gao and Serrero, 2000). The expression of ADRP was identified in early stage of differentiation for adipocyte cells (Jiang and Serrero, 1992) and pressed abundantly in the liver (Brasaemle *et al*., 1997; Jiang and Serrero, 1992). Overexpression of ADRP increased triglyceride accumulation while knockdown of ADRP decreased the pool of cytosolic lipid droplets (Magnusson *et al*., 2006). ADRP has been regarded as a sensitive marker of lipid loading in human blood monocytes and in human monocyte-derived macrophages (Llorente-Cortes *et al*., 2007). In pigs, the *ADRP* gene was identified to locate on chromosome 1 q2.3-2.7 between microsatellite markers SW2185 and SW974 using a three generation Korean reference family (Kim *et al*., 2005). It has been reported that QTL affecting growth and fat deposition traits in this region contained the porcine ADRP locus (Rohrer and Keele, 1998; De Koning *et al*., 1999; Bidanel *et al*., 2001; Quintanilla *et al*., 2002). Taking together, the biological role of this gene and the mapping localization indicated that the porcine ADRP is a possible candidate gene for fat deposition in pig breeding. Indeed, in the present study, the ADRP genotypes showed significance both on leaf fat weight and water moisture ($p<0.05$). Similar effects were also detected in chicken (Zhao *et al*., 2009).

CONCLUSION

In this study researchers could not determine whether the association is a direct effect or the effect of a tightly linked QTL due to the extensive linkage disequilibrium in the F2 hybrids. For a better evaluation of the presence of their effects on fat deposition, increasing the number of pigs and records from other populations and pig breeds would be required.

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