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Expression and genome polymorphism of *ACSL1* gene in different pig breeds

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Abstract Acyl coenzyme A long-chain 1 synthetase (ACSL1) plays a key role in animal fat synthesis and fatty acid β-oxidation. In order to research the function of the *ACSL1* gene in pig, we analyzed the mRNA expression in liver, backfat and *longissimus dorsi* muscle by quantitative real-time PCR in Tibet pig (TP, $n = 10$), Diannan small ear pig (DSP, $n = 10$) and large white pig (LW, $n = 10$). The results showed that the mRNA expressions of the *ACSL1* gene in liver and *longissimus dorsi* muscle of DSP and TP were significant higher than that of LW ($P < 0.01$). However, the expression in backfat of LW was significant higher than that of TP ($P < 0.01$) and DSP ($P < 0.05$). In addition, four SNPs located in 5′ flanking region (*T-1191C*), exon 6(*G173A*), exon 14(*C36T*) and exon 17(*T46C*) were identified, and the allele frequencies of the four SNPs were significant different in indigenous and introduced pig breeds. The results indicated that the *ACSL1* gene might be relative to the capacity of fat deposition and meat quality in pig breeds.

Keywords ACSL1 · mRNA expression · Polymorphism · Adipose deposition · Pig

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Introduction

Lipid deposition in pig is a very complex trait that is likely to be controlled by many genes [1] and is determined by a complex balance between lipogenesis and lipolysis [2–4]. In the balance, fatty acids are intermediate products and must be activated to acyl coenzyme A before participating in most catabolic and anabolic reactions [5]. Through investigation in last decades, it was confirmed that the acyl coenzyme A synthetase (ACS) was the main enzyme to catalyze the activation reactions. ACS family includes multiple isoforms classified by their substrate specificities for fatty acids of varying chain length [6]. In mammals, long-chain acyl-CoA synthetase (ACSL) catalyzes the ATP-dependent acylation of fatty acids into long-chain acyl CoAs, which is the first step in lipid metabolism after fatty acid entry into the cell [7]. The ACSL isoform, an important ACS family member, plays an essential role in both lipid biosynthesis and fatty acid degradation [8], and also plays regulatory roles in numerous reactions, including, for example, protein modification [9], intracellular protein transport [10], protein kinase C activation [11], nuclear thyroid hormone receptor modulation [12], and cell proliferation [13].

There are five cloned isoforms of ACSL: ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 [9]. These isoforms are expressed not only specially in some certain tissues, but also differently at some certain developing ages. ACSL1, ACSL4, and ACSL5 are all found in liver and adipocytes [14–16], whereas both of ACSL3 and ACSL6 are expressed in brain [17, 18]. ACSL4 expression is most abundant in steroidogenic tissues and ACSL5 in intestine [14, 15]. After birth of rats, just the *ACSL1* expression increased by fourfold in heart, whereas the *ACSL3* decreased and the other ACSL isoforms remained stable [19]. Furthermore,

during 3T3-L1 adipocyte differentiation, the expression of *ACSL1* gene increases by about 160-fold, while the expression of other isoforms is unchanged [20]. Transgenic mice with overexpressed *ACSL1* in heart increased TAG content [21]. Furthermore, overexpression of *ACSL1* mRNA and protein by more than fivefold over controls caused a twofold increase in TAG content in mouse liver [22]. So the *ACSL1* overexpression has convincingly been thought to increase TAG accumulation.

The pig *ACSL1* gene has been mapped on chromosome 15, near the SW1989 microsatellite [23]. It is split into 20 exons and the resulting cDNA encompass 3,133 bp, of which 2,097 bp correspond to the coding region. However, it is unclear whether the trait of fat accumulation in pigs is relative to the *ACSL1* gene expression in some organs. We proposed a hypothesis that the varying phenotype of fat traits in pig might be due to different quantity of the *ACSL1* expression or polymorphism of SNPs in *ACSL1* gene region. The aim of this study was to investigate the differences of *ACSL1* expression and genome polymorphisms in several pig breeds that have different intramuscular fat (IMF) contents, and to provide basic molecular information for the further research on the function of the *ACSL1* gene in pig.

Materials and methods

Experimental materials

This experiment covered three breeds of pig: Tibet pig (TP) from Tibet Agricultural and Animal Husbandry College, Diannan small ear pig (DSP) and large white (LW) from Xishuang Banna city, Yunnan province, China. 30 castrated boars (10 each group) were slaughtered when they were 6-month age. Tissue samples were collected from liver, backfat, *longissimus dorsi* muscle at the last rib. The samples were immediately frozen in liquid nitrogen, and then were stored at −80 °C. Ear samples were collected from populations of TP ($n = 67$), DSP ($n = 54$) and LW ($n = 56$) which was used to detect SNPs in the region of *ACSL1* gene.

DNA, RNA extraction and cDNA preparation

Genomic DNA were isolated from ear tissues with the extraction procedure as described [24], dissolved in TE solution and preserved in −20 °C refrigerator.

Total RNA was isolated from the liver, backfat and *longissimus dorsi* muscle tissues with TRIZOL® Reagent (Invitrogen, San Diego, CA, USA) using the method of the manufacturer's instructions. The RNA solutions were checked for concentration and purity using an NanoDrop 2000 Biophotometer (Thermo scientific, USA) at 260/280 nm absorbance ratio (range 1.8-2.0 indicates a pure RNA sample) and in a 1 % agarose gel to verify its integrity.

After treatment with DNase I, the total RNA was reverse transcribed to cDNA in a reaction volume of 20 μL containing 2 μg total RNA, 50 μmol oligo-d(T) $_{15}$ as a primer and 10 nmol dNTP mix. These mixtures were heated at 70 °C for 5 min and incubated on ice for 2 min. After that, 200 U ImProm-II^{TM} reverse transcriptase (Promega, USA), 40 U RNase inhibitor (Promega Biotech Co., Ltd.) and 4 μL 5× reaction buffer were added to the each mixture and was incubated at 42 °C for 60 min, and then inactivated by heating at 70 °C for 15 min.

Quantitative analysis of ACSL1 mRNA expression

The expression quantity of *ACSL1* gene was measured using real-time PCR. Primers were designed using Primer Premier 5.0 software spanning one intron to avoid genomic DNA contamination. *ACSL1* (NM_001167629) primers were: 5′-GCA GGC ATT TCT CAT AGC G-3′ and 5′-TCC CTC CCC AGT CTC AGC AT-3′. We selected the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal standard [25]. GAPDH primers were 5′-GGT CAC CAG GGC TGC TTT TA-3′ and 5′-CCT TGA CTG TGC CGT GGA AT-3′. Real-time PCR amplification was conducted using Bio-Rad CFX96 System (Bio-Rad, USA). The gene expression quantity was calculated with the method of $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_{target\ gene} - \Delta Ct_{housekeeping\ gene}$) [26]. A cDNA pool of all samples was used as a calibration.

SNP screening

Primers for SNP identification were designed using Primer Premier 5.0 software allowing the amplification of a region of 5′ flanking region and coding region (from exon 1 to 20). The targeted regions, primer sequence and the amplicon size are shown in Table 1. The PCR products amplified from 10 pigs each group were pooled and sequenced directly to identify SNPs. Chromas Pro and DNAMAN6.0 were used to analyze the sequencing results.

SNP genotyping

For SNPs found in sequence alignment, the software of NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) was used online to search for special restriction enzymes. The *HinfI*, *HhaI*, *PvuII*, *TasI* were selected for genotyping loci of 5′ flanking *T-1191C,* exon 6 *G173A,* exon 14 *C36T* and exon 17 *T46C* respectively. The reaction conditions were shown in Table 2.

Table 1 Sequence, targets and amplicon size of the primers used for SNP identification of the pig *ACSL1* gene

Primer	Target region	Forward primer sequence (5′ to 3′)	Reserve primer sequence (5′ to 3′)	Amplicon size (bp)
5′-FR1	−2, 133/−1, 698 bp	GGTTTGTTGAATGGCTGGAGTA	CTCGGTCTCTCTGGGCACTCAT	436
5′-FR2	−1, 812/−999 bp	CATCCACCGTCTCTTCTCCT	AATCAGATAAACAACCCCAT	813
ACSL-P1	Exon 1	GTTTCTGCCTCTCTGTTCTTCT	CGTTTGCCTGTGATTGTGA	654
ACSL-P2	Exon 2	TCTTGTTGCTCGGTCTCCCTTA	AAGTTACAGAGGAGACAGGGGACC	431
ACSL-P3	Exon 3	CCATAGTAGTGTCTGCCTGAT	ATGTCCAAACACTAAGGCTC	233
ACSL-P4	Exon 4	TCAGTTTCCTTACAGTGCGT	AGAACTCTCCACACTACACG	358
ACSL-P5	Exon 5–6	CAGGTTCAGAATCCCTCGTG	GCGACTTGCTTGATGAGAAC	877
ACSL-P6	Exon 7–8	ATGAGAGGCAAGGGCGTGAGCA	CACAAAAGGAGGCAGGGGAT	646
ACSL-P7	Exon 9	CATCCTCTAACTTGGTTCTGCT	CAGAATCCAAAAGAGGGTCACT	393
ACSL-P8	Exon 10	CACCTCCTTTCCCTTTACGA	GACTTAGAACACAGACAGGCGA	373
ACSL-P9	Exon 11	GTTGTATGTTGTCTGTGCGTTGT	GGGCGGTGATGGACTCTGGTT	372
ACSL-P10	Exon 12	TCCCCATCTTCACCAGCACTTGT	TTGCTGTGACTGTGGCATAGGC	644
ACSL-P11	Exon 13	CTGGGCGTCCTTGCCTGTTAGT	TGTGCCTACATCGCCTTCTCCA	385
ACSL-P12	Exon 14	CCTGCTCCTCCTCTTCCTTCT	AAATCACATCCCGCTCCACCA	282
ACSL-P13	Exon 15	TCTGAGTAGGCTGGTTTGTGAT	GAGAAGTGGCCCTGCTATGAAG	239
ACSL-P14	Exon 16	TCAGGTGTGTGTGAAAGGGC	GAGGAGAGAAAGTTAGGGAGC	214
ACSL-P15	Exon 17	GCAAGGTATTTGTCCCTCTAAGC	GTTGGCTTGGAACCACTATTTGC	941
ACSL-P16	Exon 18–19	CGAGCCGTGTCATCTCTCATTC	CTCTTCCAGAACCTATTCGCCA	443
ACSL-P17	Exon 20	TAACCGCTGAGCCACGAAGGGAC	GCCCTCTCCTGTTCCTATGACG	355

Table 2 Restriction enzyme digestion loci and reaction conditions

Enzyme name	Loci	Incubate temperature (°C)	Reaction time (h)
*Hin*f I	5′ flanking *T-1191C*	37	8
*Hha*I	Exon 6 *G173A*	37	8
*Pvu*II	Exon 14 *C36T*	37	8
*Tas*I	Exon 17 *T46C*	65	8

Statistical analysis

The expression levels were analyzed by one-way ANOVA with repeated measures using SAS9.1 software (SAS Inst. Inc., Cary, NC). The results are presented as mean ± standard error. Significant and extreme differences were set at $P < 0.05$ and $P < 0.01$, respectively. χ^2 test was used to analyze the distribution of genotypes and the differences in alleles frequencies.

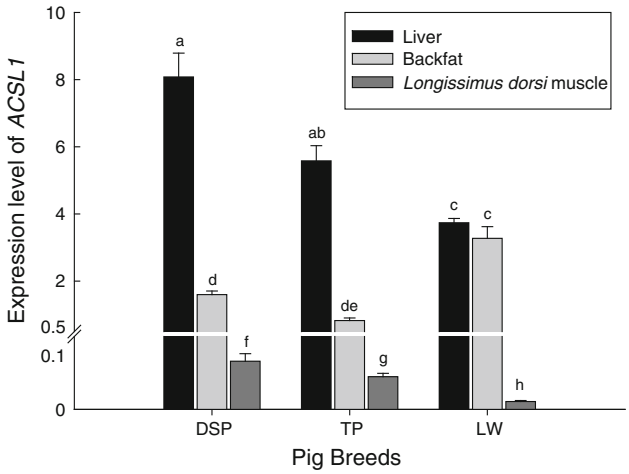

Fig. 1 The *ACSL1* mRNA expression level in three tissues of the three pig breeds. *Note Error bars* represent SE. *Letters on bars* denote the difference of expression level with significantly difference ($P < 0.05$). Break range on *Y* axis is 0.1–0.5 being omitted. *DSP* diannan small ear pig ($n = 10$). *TP* Tibet pig ($n = 10$), *LW* large white ($n = 10$)

Results

ACSL1 mRNA expression level in the three tissues among the three breeds of pig

The *ACSL1* mRNA expression level was different among breeds and tissues as shown in Fig. 1. In both DSP and TP, the *ACSL1* gene expression was maximal in liver tissue and mid in backfat and minimal in *longissimus dorsi* muscle, and the differences among the three tissues were extremely significant differences ($P < 0.01$). But in LW, the expression difference between liver and backfat tissue was not significant ($P > 0.05$). The *ACSL1* expression level in liver tissue of DSP and TP was significantly higher than that of LW ($P < 0.01$), and there was no significant differences of the gene expression in liver between DSP and TP

Table 3 Genotype frequency and gene frequency of the four SNPs in three pig breeds

Loci	Breed	Genotype frequency (number/frequency)				Allele gene frequency	
		TT	TC	CC	χ^2 value (P value)	T	C
T-1191C	TP	41/0.6119	19/0.2836	7/0.1045	1.751 ($P = 0.417$)	0.7537	0.2463
	DSP	38/0.7037	15/0.2778	1/0.0185	0.023 ($P = 0.989$)	0.8426	0.1574
	LW	56/1	0/0	0/0	–	1	0
		GG	GA	AA	χ^2 value (P value)	G	A
G173A	TP	18/0.2686	28/0.4179	21/0.3134	0.906 ($P = 0.636$)	0.4776	0.5224
	DSP	9/0.1667	10/0.1852	35/0.6481	7.353 ($P = 0.025$)	0.2593	0.7407
	LW	0/0	0/0	56/100	–	0	1
		CC	CT	TT	χ^2 value (P value)	C	T
C36T	TP	28/0.4179	28/0.4179	11/0.1642	0.419 ($P = 0.811$)	0.6269	0.3731
	DSP	35/0.6481	11/0.2037	8/0.1481	6.315 ($P = 0.043$)	0.7500	0.2500
	LW	56/100	0/0	0/0	–	1	0
T46C		TT	TC	CC	χ^2 value (P value)	T	C
	TP	23/0.3433	31/0.4627	13/0.1940	0.125 ($P = 0.940$)	0.5746	0.4254
	DSP	29/0.6481	22/0.2037	3/0.1481	0.101 ($P = 0.951$)	0.7407	0.2593
	LW	47/100	9/0	0/0	0.05 ($P = 0.975$)	0.9196	0.0804

($P > 0.05$). However, the gene expression in backfat tissue of LW was higher than that of DSP and TP ($P < 0.05$), and no significant difference was seen in backfat of DSP and TP ($P > 0.05$). The expression level in *longissimus dorsi* muscle was the highest in DSP, and was higher than in TP and LW ($P < 0.05$). And the expression was significantly higher in TP than in LW ($P < 0.05$).

SNP identification

Four SNPs, 5′ flanking region (*T-1191C*), exon 6 (*G173A*), exon 14 (*C36T*), and exon 17 (*T46C*), were identified by sequencing. The mutations of exon 6 *G173A*, exon 14 *C36T* and exon 17 *T46C* caused no amino acid changes, so they were all synonymous mutations. No restriction enzymes were found to identify the locus of 5′ flanking region *T-1191C*, so we designed a forward mismatched primer for identify the mutation using the dCAPS Finder 2.0 software (http://helix.wustl.edu/dcaps/dcaps.html). The mismatched primer sequence was 5′-CAA AAA TAT CAT CTC AAC TTA GAG T-3′, which was used for PCR-RFLP analyzing for the *T-1191C* with the reverse primer of 5′-*FR2* (listed in Table 1). PCR product size of the primers was 218 bp, which could be digested by restriction enzyme *Hin*f I on the loci of *T-1191C*. The PCR products amplified using the primers *ACSL-P5* and *ACSL-P12* listed in Table 1 respectively were used to be digested by *Hha*I and *Pvu* II to genotype of the locus of exon 6 *G173A* and exon 14 *C36T*. Another forward primer was designed for amplification of exon 17 region with the reverse primer of *ACSL-P15* listed in Table 1, and the sequence was 5′-ACA GGC TCA CTT CGC AGG TAG AT-3′. And the PCR product (292 bp) was digested by *Tas*l to detect the genotype of the exon 17 *T46C*.

SNP genotype frequency

The genotype frequency and allele frequency of each site of *ACSL1* gene in TP ($n = 67$), DSP ($n = 54$) and LW ($n = 56$) breeds were shown in Table 3. The results showed that polymorphisms of *T-1191C*, *G173A*, and *C36T* were only identified in TP and DSP populations, whereas polymorphism of *T46C* was found in the three breed populations. The χ^2 test showed that four locus were staying in Hardy–Weinberg equilibrium ($P > 0.05$) in TP population. But in DSP and LW populations, only the *T46C* site was staying in Hardy–Weinberg equilibrium ($P > 0.05$). The C allele frequency of *T-1191C* site and the T allele frequency of *C36T* site in TP population were higher than those in DSP ($P > 0.05$), and the G allele frequency of *G173A* site in TP population was significantly higher than that in DSP ($P < 0.01$). In *T46C* site, the C allele frequencies of TP and DSP populations were extremely significant higher than that of LW ($P < 0.01$).

Discussions

The native breeds such as TP in the Qinghai-Tibet Plateau and DSP in south of Yunnan province of China have good adaptation to the harsh conditions and have superior meat quality [27–29]. The two indigenous pigs have high IMF content, whereas LW, a introduced pig breed, possesses good performances in growth rate and lean ratio, but has a low IMF [29, 30]. The *ACSL1* expression level was 1.8- and 2.2-fold higher in livers of TP and DSP respectively, and was 4- and 6-fold higher in *longissimus dorsi* muscles of TP and TSP respectively, comparing with LW. The data in vitro and in vivo had indicated that the ACSL1 was linked to the storage pathway of lipid metabolism in liver and that it might act to channel fatty acids into triglyceride synthesis rather than into β-oxidation and energy production [22]. Our data suggest that the DSP and TP breeds with higher capacity of adipose deposition need more ACSL1 to synthesize long-chain acyl-coenzyme A for the synthesis of triglyceride, which may be the reason of higher IMF content in TP and DSP breeds than in LW. Otherwise, the expression level in backfat of LW was higher than that in TP and DSP. Those results suggested that the expression of *ACSL1* in different tissues may be regulated by different transcription factors. For instance, *ACSL1* in liver is regulated by *PPARα* [31, 32] that mainly expressed in liver [33, 34], whereas it is regulated by *PPARγ* [35] in adipose tissue [36, 37]. Otherwise, it is also possible that more ACSL1 needed for β-oxidation in backfat of LW than the other breeds. The partitioning of intracellular fatty acids between storage pathways and β-oxidation is also controlled by regulation of the mitochondrial acyl-CoA transporter carnitine palmitoyltransferase-1 [38].

In this study, we identified four SNPs in the region of pig *ACSL1* gene. The 5′ flanking region *T-1191C*, exon 6 *G173A* and exon 14 *C36T* sites had not been reported in previous works and the polymorphisms of the three locis were only found in Chinese indigenous pig breeds. For the 5′ flanking *T-1191C*, the binding sites of transcription factors were predicted by the software CONSITE online (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite). The results indicated that the mutation of T → C led sites for combining with SOX17 and broad-complex-1 transcription factors. The SOX17 transcription factor mainly acts as a regulator for sustaining self-renewal of hematopoietic stem cells [39, 40]. The broad-complex-1 is a zinc-finger protein found in insects such as *Bombyx mori*, *Drosophila melanogaster*, and it has not been found the homologisation sequences in mammals. No polymorphisms of the exon 6 *G173A* and exon 14 *C36T* sites were found in LW and also in Duroc, Landrace, Piétain, LW and Iberian populations [41]. So, we suggested that the mutations of the two sites may be particular to the TP and DSP populations. The polymorphism of exon 17 *T46C* was detected in the three pig breeds and in other breeds such as Duroc, Landrace [41], but the mutation in exon 17 region was not found in Piétain and Iberian populations [41].

In conclusion, the pig *ACSL1* gene expresses differently in different tissues and pig breeds. The acyl coenzyme A activated by ACSL1 can enter any metabolic both lipid biosynthesis and fatty acid degradation [5], the mechanism of higher IMF content in fatty-type pigs may be due to more ACSL1 needed for lipogenesis in liver and *longissimus dorsi* muscle tissues, and the thinner of backfat thickness in lean-type pigs may be due to more ACSL1 needed for β-oxidization in backfat. We found four mutant loci in the two indigenous pigs, but only one of them was multiple in LW populations. The allele frequencies of the four sites were significantly different in indigenous and introduced pig breeds. Those four mutations may be relative to fat deposition in backfat and IMF. However, further studies will be needed such as correlation analysis between *ACSL1* expression level and the traits of backfat thickness and IMF content, genotypes of SNPs and lipid deposition traits.

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