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ASCL2 Gene Expression Analysis and Its Association with Carcass Traits in Pigs

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ABSTRACT : Achaete-scute like 2 (*ASCL2*) gene encodes a member of the basic helix-loop-helix transcription factor which is essential for the maintenance of proliferating trophoblasts during placental development. *ASCL2* gene preferentially expresses the maternal allele in the mouse. However, it escapes genomic imprinting in the human. In this study, the complete open reading frame consisting of 193 amino acids of *ASCL2* gene was obtained. Sequence analysis indicated that a C-G mutation existed in the 3' untranslated region between Meishan and Large White pigs. The polymorphism was used to determine the monoallelic or biallelic expression with RT-PCR-RFLP in pigs of Large White×Meishan F_1 hybrids. Imprinting analysis indicated that the *ASCL2* gene expression was biallelic in all the tested tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, fat, uterus, ovary and pituitary). PCR-RFLP was used to detect the polymorphism in 270 pigs of the "Large White×Meishan" F_2 resource population. The statistical results showed highly significant associations of the genotypes and fat meat percentage (FMP), lean meat percentage (LMP) and ratio of lean to fat (RLF) ($p<0.01$), and significant associations of the genotypes and loin eye area (LEA) and internal fat rate (IFR) ($p<0.05$). (**Key Words :** *ASCL2*, Imprinting, Porcine, Carcass Traits, Polymorphism)

INTRODUCTION

Genomic imprinting is a parent-of-origin-dependent epigenetic mechanism in which a subset of autosomal genes are expressed from only one allele (Smith et al., 2003). Imprinted genes have an important function in the regulation of fetal growth, development, function of the placenta and postnatal behavior, particularly in mammals (Reik et al., 2003). At present, more than 120 imprinted genes have been identified in the human and mouse, but only 10 imprinted genes have been identified in sheep, 7 in cattle and 3 in pigs (http://igc.otago.ac.nz/home.html). Therefore, it is of interest to analyze the status of imprinted genes and their effect on growth and carcass traits in pigs.

Achaete-scute like 2 (*ASCL2*) gene has been located in the center of the 11p15 imprinted domain which contains the imprinted genes *TSSC3*, *TSSC5*, *IGF2*, *H19* and so on (Lee et al., 1999). In the mouse, *ASCL2* gene was maternally expressed (Guillemot et al., 1995), but Miyamoto et al. (2002) reported that the gene escaped genomic imprinting in the human. Stepan et al. (2003) showed that the *ASCL2* gene was essential for adequate differentiation of the trophoblast and disruption of it leads to early intrauterine death. Jubb et al. (2006) proved that the *ASCL2* gene was essential for the maintenance of proliferating trophoblasts during placental development.

In the present study, we obtained the complete coding region and detected the imprinted status of the porcine *ASCL2* gene with RT-PCR-RFLP using a C-G mutation which existed in the 3' untranslated region. Association analysis between the polymorphism and carcass traits was tested in 270 pigs of the Large White×Meishan F_2 resource population.

MATERIALS AND METHODS

Experimental animals

All animals in this study were derived from the experimental pig station of Huazhong Agricultural University. An adult Large White and an adult Meishan pig were used to search for SNP. Twelve two-month old pigs of

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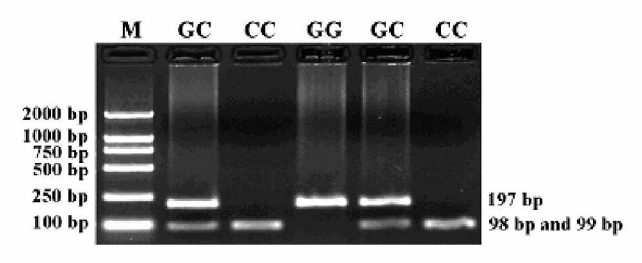

Figure 1. PCR-*Msp*I-RFLP of porcine *ASCL2* gene.

Large White×Meishan F_1 hybrids and their mothers were used for imprinting analysis. Pigs of Large White×Meishan F_2 hybrids used for association analysis were fed as described by Dai et al. (2006). The finishing animals were slaughtered in the year of 2003 and 2004 and measured in the Center of Swine Control of China (Wuhan) according to the method of Xiong and Deng (1999). Genomic DNA was isolated according to the standard phenol-chloroform method.

RNA isolation and cDNA synthesis

Total RNA, from the skeletal muscle tissue of the adult Large White and Meishan pigs and from twelve tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, fat, uterus, ovary and pituitary) of four heterozygous pigs (based on SNP) of the twelve F_1 hybrids, was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions. The first strand cDNAs were synthesized from 3 μg total RNA treated with DNAse I (TaKaRa, Tokyo, Japan) in a 25 μl reaction containing 40 U M-MLV reverse transcriptase, 5 μM oligo$(dT)_{18}$ primer, 1×M-MLV first-strand buffer, 1 mM each of dNTP and 8 U RNase inhibitor (Promega, Madison, WI, USA) at 42°C for 60 min.

PCR of DNA and cDNA

Human *ASCL2* cDNA sequence (GenBank: NM_005170) was used to identify pig expressed sequence tags (EST) through standard BLAST (http://www.ncbi.nlm.nih.gov/blast/) searches of the 'EST-others' database. Pig ESTs sharing more than 85% sequence identity with the human cDNA sequence were assembled into an EST-contig. The exon-intron structures of the porcine *ASCL2* gene were estimated according to the human *ASCL2* gene. Primer pair ASF/ASR (ASF: ACAGTGCCAGATCGTCGCT and ASR: TGTCCCTGGGCAGTTCAAG) was designed according to the EST-contig. Primer pair ASDF/ASDR (ASDF: CCTGACCAAGGGCTAGTG and ASDR: GTCCCTGGG CAGTTCAAG) was used to detect SNP clearly. PCR conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 45 s, 60°C (ASF/ASR) or 56°C (ASDF/ASDR) for 50 s, 72°C for 90 s (ASF/ASR) or 35 s (ASDF/ASDR) and a final extension at 72°C for 7 min. Primers F: ACCA CAGTCCATGCCATCAC and R: TCCACCACCCTGTT GCTGTA, which amplified the fragment spanning intron 8 of the *GAPDH* gene, were applied to exclude the possibility of DNA contamination during all RT-PCR reactions.

Sequencing and SNP detection

PCR products were purified with the Wizard prep PCR purification system (Promega), cloned with pMD18-T easy vector (TaKaRa) and sequenced commercially. Sequences of Large White and Meishan pigs were compared with DNAStar software to search for SNP.

RFLP of PCR and RT-PCR products

PCR or RT-PCR products (6 μl) amplified by primer pair ASDF/ASDR were incubated at 37°C for 4 h with 3 U restriction enzyme *Msp* I (TaKaRa), 2.7 μl purified water and 1 μl digestion buffer. Digested products were separated by 2% agarose gel.

Statistical analysis

The association between genotype and carcass traits was performed with the least square method (GLM procedure, SAS version 8.0). According to the method of Liu (1998), both additive and dominance effects were also estimated using REG procedure of SAS version 8.0, where the additive effect was denoted as -1, 0 and 1 for GG, GC and CC, respectively, and the dominance effect represented as 1, -1 and 1 for GG, GC and CC, respectively. The statistical model was assumed to be: $T_{ijk} = \mu+S_i+Y_j+G_k+b_{ijk}X_{ijk}+e_{ijk}$, where T_{ijk} is the observed values of traits; μ is the least-square mean; S_i is effect of sex (i = 1 for male or 0 for female), Y_j is the effect of year (j = 1 for year 2003 or 0 for year 2004), G_k is the effect of genotype (K = GG, GC and CC), b_{ijk} is the regression coefficient of the slaughter weight, X_{ijk} is the slaughter weight, and e_{ijk} is the random residual.

RESULTS

Sequence analysis and SNP discovery

Primer pair ASF/ASR amplified a 1,654 bp fragment (Genbank accession number DQ666420) in genomic DNA. Aligning the sequence with human *ASCL2* gene cDNA sequence showed it including 117 bp of 5' untranslated sequence, 582 bp of coding sequence and 955 bp of 3' untranslated sequence. Sequence analysis indicated that it had 84% and 81% sequence similarity in nucleotides or 84% and 79% sequence similarity in amino acids compared to the homology of human and mouse, respectively. Compared sequences of Large White and Meishan breeds revealed one SNP (C/G) which existed in the 3' untranslated region at position 1,556 (DQ666420).

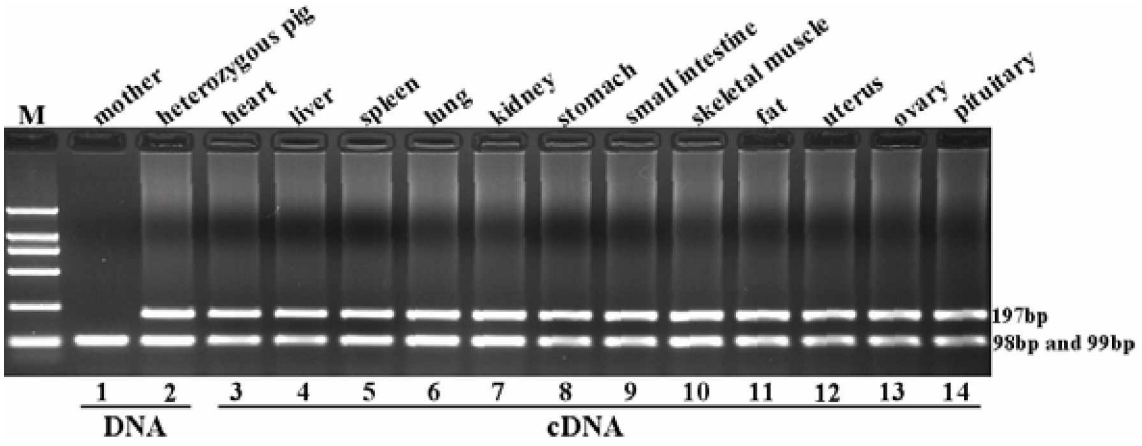

Figure 2. Tissue expression of porcine *ASCL2* gene. Allelic expression of *ASCL2* by PCR-RFLP. The cDNA samples were from 12 different tissues of a heterozygous pig (lanes 3-14), and genomic DNA samples were from the pig (lane 2) and its mother (lane 1) Digestion with *Msp*I revealed that expression of the *ASCL2* gene was biallelic in all the tested tissues. M: Mark DL-2000 (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp).

Table 1. Association of *ASCL2* genotypes and carcass traits

Trait[1]	Genotype (Lsmean±SE)[2]			Effect (mean±SE)	
	GG (n = 32)	GC (n = 110)	CC (n = 128)	Additive	Dominance
LMP (%)	0.484±0.009[A]	0.522±0.004[B]	0.524±0.004[B]	-0.0191±0.0032**	-0.0069±0.0022**
SFT (cm)	3.934±0.222	3.894±0.107	3.870±0.105	0.0005±0.0743	0.0121±0.0499
RFT (cm)	3.248±0.151	2.968±0.073	2.924±0.072	0.0258±0.0615	0.0253±0.0413
TFT (cm)	2.503±0.151	2.295±0.073	2.183±0.071	0.0198±0.0604	-0.0080±0.0406
BFT (cm)	2.322±0.189	2.089±0.092	2.034±0.090	-0.0044±0.0708	0.0398±0.0476
ABT (cm)	3.002±0.146	2.811±0.071	2.753±0.069	-0.0319±0.0598	-0.0022±0.0402
LEA (cm^2)	25.604±1.154[a]	27.319±0.559	28.726±0.546 b	-1.5893±0.5079**	0.2274±0.3413
CL (cm)	89.533±1.159	90.348±0.562	90.553±0.549	-1.1846±0.6710	-0.0679±0.4510
FMP (%)	0.291±0.012[A]	0.248±0.005[B]	0.244±0.005[B]	0.0142±0.0045**	0.0071±0.0030**
RLF	1.704±40.149[A]	2.200±0.072[B]	2.222±0.071[B]	-0.2122±0.0672**	-0.0973±0.0451*
IFR (%)	0.038±0.002[a]	0.034±0.001	0.034±0.001[b]	-0.0001±0.0007	-0.0004±0.0005

[1] FMP = Fat meat percentage; LMP = Lean meat percentage; IFR = Internal fat rate; 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. CL. Carcass length; LEA. Loin eye area.

[2] Least square mean values (±SE).

[3] Different letters denoting significant difference between groups: [a, b, *] $p<0.05$.

Screening for heterozygous pigs

The C-G mutation could be detected by restriction enzyme *Msp* I. Primer pair ASDF/ASDR which amplified fragment covered the SNP was designed to distinguish the genotypes well. Allele C was 98 bp and 99 bp, and allele G was 197bp (Figure 1). Twelve F_1 hybrids of Large White and Meishan pigs were used to choose heterozygous individuals at the SNP. Amplification of primer pair ASDF/ASDR was conducted with the genomic DNA of the twelve pigs and their mothers. PCR products were digested by *Msp* I. Results of PCR- *Msp* I -RFLP indicated that four pigs of Large White and Meishan F_1 hybrids were heterozygous at the SNP.

Imprinting analysis of *ASCL2* gene

RT-PCR of heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, fat, uterus, ovary and pituitary from the four pigs with primer pair ASDF/ ASDR showed that the *ASCL2* gene was expressed in all tissues. RFLP analysis of the RT-PCR products showed that the gene expression was biallelic in all the examined tissues (Figure 2).

Association analysis of genotypes and carcass traits

Two hundred and seventy pigs of the Large White×Meishan F_2 resource family were used to estimate the association between the polymorphism and carcass traits. At this locus, the number of genotypes GG, GC and CC were 32, 110 and 128 respectively, and the genotype distribution conformed to the Hardy Weinberg Equilibrium ($\chi^2 = 1.222 < \chi^2_{(0.05)}$, d.f. = 2). The detailed results of association analysis are listed in Table 1. Statistical results

showed that the PCR-*Msp* I -RFLP genotypes had highly significant association with fat meat percentage (FMP), lean meat percentage (LMP) and ratio of lean to fat (RLF) ($p<0.01$), and significant associations with internal fat rate (IFR) and loin eye area (LEA) ($p<0.05$). At this locus, additive and dominance both seemed to be highly significant ($p<0.01$) and allele C was associated with increases in the trait value. Pigs with the CC genotypes had higher lean meat percentage (4%) and more loin eye area (3.122 cm^2).

DISCUSSION

The *ASCL2* gene was maternally expressed in the mouse (Guillemot et al., 1995), but Miyamoto et al. (2002) reported biallelic expression in the human. In pigs, expression was also biallelic in various tissues of the four heterozygous pigs. Similar imprinted status between pigs and humans showed that the two species were adjacent in genomic imprinting, which also confirms the conservation of genomic imprinting between the two species. Sequence analysis showed 84% and 81% sequence similarity in nucleotides and 84% and 79% sequence similarity in amino acids compared to human and mouse homology, respectively. The high sequence similarity in nucleotides and amino acids between humans and pigs showed they may have similar biological function. The different imprinted status in the human, mouse and pig indicated that different imprinting mechanism or imprinting regulation may exist among them.

Imprinted genes have an important function in the regulation of fetal growth, development, function of the placenta and postnatal behavior in mammals (Reik et al., 2003). In previous studies, many imprinted QTLs affecting porcine carcass composition and growth traits were found in several chromosomes (Andersson et al., 1994; Alexander et al., 1996; Nezer et al., 1999; Koning et al., 2000). *IGF2*, which was identified as the first imprinted gene in pigs, has important effects on porcine growth, meat quality and carcass composition, especially fat deposition (Jeon et al., 1999; Jungerius et al., 2004; Estelle et al., 2005). Zhang et al. (2006) reported that the *PLAGL1* gene which was imprinted in the human and mouse had important effects on shoulder backfat thickness (SFT) and internal fat rate (IFR). In addition, *ASCL2*, *H19* and *IGF2* were associated with an imprinted QTL in the human and mouse (Lee et al., 1999). Therefore, the porcine *ASCL2* gene was selected as a candidate gene affecting fat deposition and carcass traits in our study. The polymorphism had highly significant associations with fat meat percentage (FMP), lean meat percentage (LMP) and ratio of lean to fat (RLF) ($p<0.01$), and significant associations with internal fat rate (IFR) and loin eye area (LEA) ($p<0.05$). Our statistical results confirmed that the *ASCL2* gene had an important effect on porcine carcass traits like *IGF2*. Although the *ASCL2* gene was not imprinted in pigs in our study, its effect on porcine carcass traits was not affected by its imprinted status, which is consistent with the results that *IGF2* and *IGF2R* were not imprinted, but they also had important effects on body growth and carcass traits in chickens (Nolan et al., 2001; Wang et al., 2005). Pigs with the CC genotype had higher lean meat percentage (4%) and more loin eye area (3.122 cm^2). Increasing the allele C may be favorable for carcass traits in pig breeding. The polymorphism site could be a useful molecular marker for lean meat percentage and carcass traits.

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