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Molecular Characteristics of Porcine *SIM1* Gene and its Variants Association with Carcass and Meat Quality Traits

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Abstract: In the present study, the cDNA sequence of porcine *SIM1* gene was cloned by homology cloning and rapid amplification of cDNA ends approach. The full length cDNA of SIM1 consists of 3720 bp nucleotides. It contains an Open Reading Fame (ORF) of 2301 bp which encodes a corresponding protein of 766 amino acid residues and flanked by 461 bp 5' UTR and 958 bp 3' UTR. The porcine SIM1 protein has a high homology to that of human and cattle. Expression analysis revealed an ubiquitous distribution in all examined tissues. Two novel single nucleotide polymorphisms (SNPs) c+534 T>C and c+1677 C>T were found. Association analysis betweeen SNPs and carcass and meat quality traits were carried out in a Pietrain x Jinhua F2 population (n = 294). The results revealed extremely significant effect on average backfat thickness, intramuscular fat content, intramuscular water content, ham temperature, ham fat weight and ham lean weight ($p<0.01$) along with significant effect on loin eye area and ham pH ($p<0.05$).

Key words: *SIM1* gene, cDNA, expression, SNPs, carcass, meat quality traits, China

INTRODUCTION

The *SIM1* gene is a basic Helix-Loop-Helix/Per-Arnt-SIM (bHLH-PAS) transcription factor which is essential for the terminal differentiation of the hypothalamic neurons and strongly expressed during development of the hypothalamic-pituitary axis in the Paraventricular Nucleus (PVN) (Michaud *et al*., 1998). The hypothalamus integrates a variety of signals from brain and the periphery which have profound affects on a range of behavioral and homeostatic processes. Thus, alteration in hypothalamic function may lead to disorders ranging from autism, obesity and Prader-Willi Syndrome (PWS) to endocrine disorders (Swaab *et al*., 1995; Insel *et al*., 1999; Hollander *et al*., 2003).

SIM1 mutations are one of the few known causes of nonsyndromic monogenic obesity in both humans and mice (Tolson *et al*., 2010). Several chromosomal deletions and a balanced translocation involving chromosome 6q16-6q21 which encompasses the SIM1 locus have been identified in patients with monogenic severe obesity (Gilhuis *et al*., 2000; Varela *et al*., 2006).

Heterozygous SIM1-knockout mice developed hyperphagic with increased linear growth and enhanced sensitivity to diet-induced obesity (Michaud *et al*., 2001; Holder *et al*., 2004) while mice overexpressing SIM1 partially rescues agouti yellow and diet-induced obesity by normalizing food intake (Kublaoui *et al*., 2006). In the past decades, many candidate gene mutations had been found to be associated with carcass and meat quality traits such as backfat thickness, intramuscular fat content and meat color (Liu *et al*., 2009; Kobayashi *et al*., 2009). Thus as a selection tool, DNA test was widely used in pig industry for improvement of pork quality (Visscher *et al*., 2000). However, litter research has been performed to conducted the genetic role of *SIM1* gene on carcass and meat quality.

Porcine *SIM1* gene had been mapped at chromosome 1p13 and a C/T mutation in exon 8 had been found. Association analysis showed that this SNP had significant effect on growth, carcass composition and meat quality (Zhao *et al*., 2008). Based on the biology role of SIM1 in energy balance and the genetic studies in human and mouse, the *SIM1* gene was considered to be a promising candidate gene for carcass and meat quality traits.

The aims of current study are characterizing cDNA sequence of porcine *SIM1* gene and detecting novel Single Nucleotide Polymorphisms (SNPs) in the coding region. We further investigated the relationship between SNPs and recorded traits using a Pietrain x Jinhua F2 population.

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MATERIALS AND METHODS

Tissue collection and RNA extraction: Preliminary detection for cDNA sequence and polymorphisms of *SIM1* gene was carried out in 5 Jinhua and 5 Pietrain pigs (20 days old). Tissue samples including stomach, spleen, pancreas, heart, lung, kidney, thyroid gland, muscle, liver and hypothalamus were removed, immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol. RNA concentration was measured at 260 nm using the NanoDrop ND-100 spectro-photometer (NanoDrop Technologies, Wilmington, DE, USA). The total RNA was reverse transcribed into first strand cDNA by M-MLV reverse transcriptase (Promega, USA). The cDNA were used as template in gene cloning and expression profile analysis.

Isolation of the porcine *SIM1* cDNA fragment: Based on the human and mouse SIM1 mRNA sequence (GenBank, acc. nos. NC_005068 and NC_011376, respectively), primers (CDS-F, CDS-R) were designed using Oligo 6.0 to amplify porcine *SIM1* gene cDNA fragment (Table 1). The PCR amplification was performed in a 25 μL reactions containing 100-300 ng of cDNA isolated from Jinhua hypothalamus, 400 μM of dNTPs (Sangon, China), 0.25 μM of each primer and 1 U of Taq polymerase (TaKaRa, Japan).

The PCR procedure consisted of an initial denaturation at 94°C for 5 min followed by 36 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec with a final extension at 72°C for 10 min. The products were excised and purified after electrophoresis on a 1.5% EtBr gel using a AxyPrep™ DNA Gel Extraction Kit (Axygen Scienrific, CA, USA). The PCR purified products were ligated into the pGEM-T easy vector system (TaKaRa, Japan) and then transformed into competent *E. coil* DH5α cell. Plasmid DNA was purified and sequenced in Invitrogen Trading company (Shanghai, China) using an automated ABI3730 analyzer (Applied Biosystems, CA, USA).

Rapid amplification of 3' and 5' cDNA ends: Gene-Specific Primers (GSP) and Nested Gene-Specific Primers (NGSP) were designed based on the obtained cDNA fragment. 5'-RACE reactions were performed using SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA). Total RNA were reverse transcribed with SMARTer αA oligo into RACE-Ready cDNA. GSP1 and 10× Universal Primer A Mix (UPM) were used for 5'-RACE. The PCR was performed using the following condition: 5 cycles with 94°C for 30 sec, 72°C for 30 sec then 5 cycles with 94°C for 30 sec, 70°C for 30 and 72°C for 2 min at last 25 cycles with 94°C for 30 sec, 68°C for 30 and 72°C for 2 min.

3'-RACE experiments were carried out with 3'-Full RACE Core Set (TaKaRa, Japan) according to the manufacture's protocol. About 4 μg of total RNA isolated from hypothalamous was reverse transcribed into first-strand cDNA using the adaptor primer. 3'-RACE OUTER primer and GSP2 were used for outer PCR, 3'-RACE INNER Primer and NGSP2 were used for nested PCR. 3'-RACE PCR profile were 94°C for 3 min, followed by 20 cycles for GSP2 or 30 cycles for NGSP2 with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, 1 min at 72°C and a final extension of 10 min at 72°C. 3'-RACE and 5'-RACE PCR products were gel-purified and sequenced as mentioned above.

Sequence analysis: The Open Reading Frame (ORF) was found using the DNA star software and the nucleotide

Table 1: Primers designed for porcine *SIM1* gene

Primer names	Primer sequence (5'-3')	Function	T_m (°C)
CDS-F	TTTTCTGGTCGCCGAGTT	cDNA fragment	62
CDS-R	TGGAGGCTGCTGGTAGTTTG		
GSP1	CATGGTGAAGTGTTGGCAAGA		68
UPM	CTAATACGACTCACTATAGGGC		
	AAGCAGTGGTA 5'-RACE TCAACGCAGAGT		
GSP2	GATCCACGGGCGAGGTCATT	3'-RACE outer PCR	55
OUTER	TACCGTCGTTCCACTAGTGATTT		
NGSP2	CCGAGCAGTATCAAAGTAGCCC	3'-RACE inner PCR	55
INNER	CGCGGATCCTCCACTAGTGATTTCACTATAGG		
EX-F	TGAAGATCCGACAGTACAG	RT-PCR	60
EX-R	GGAGTCCAGGAAGATGAG		
β-actin-F	GCAAGTACTCTGTCTGGATTG	sqRT-PCR	60
β-actin-R	TTTGCGGTGGACAATGGA		
SIM1-1F	GCCCTACCACTCTCACTT	Detection for C534T	55
SIM1-1R	CTTCTTGTCCTGCCTCCT		
SIM1-2F	TGGGATGAAGATAGTGTGG	Detection for T1677C	55
SIM1-2R	AAGTGTTGGCAAGAGCAG		

sequences were translated into amino acids by the DNAMAN program. Phylogenetic and molecular evolutionary analysis were conducted using MEGA 3.1 (Kumar *et al*., 2004). Homologous of SIM1 proteins among different species were analyzed by DNA star software.

Tissue expression: The expressions of *SIM1* gene were detected by using semi-quantitative RT-PCR method with *α-actin* gene as endogenous control, the *SIM1* gene-specific primers (EX-F, EX-R) were designed based on the obtained cDNA sequence (Table 1). RT-PCR profile was denaturation at 94°C for 3 min, 40 cycles at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min and last extension at 72°C for 10 min. The PCR products were electrophoresis on a 1.5% EtBr gels and photographed under UV light using a Biocapt (Vilber Lourmat, France) and optical density values were analyzed using Bio-Profil Bio-2D software.

Reference population and date collection: To assess the effect of SIM1 variants on carcass and meat quality traits, the F2 resource population was constructed by the Pietrain and Jinhua pigs (PJF2) which including 4 F1 boars and 10 F1 sows and 306 F2 progeny. All 306 F2 offspring were used in the experiment, PJF2 animals were raised under normal conditions, slaughtered following electric shock at 217 days of age (SD = 30.9 days, live weight 81.3±10.55 kg) at commercial abattoir.

Four carcass traits, including: average backfat thickness (at four locations on the left carcass), loin eye area, ham fat weight, ham lean weight, along with six meat quality traits, including: intramuscular fat, protein, water content, water holding capacity, ham temperature and pH were recorded according to previous description (Zhao *et al*., 2008).

Amplification and SSCP analysis: Two novel SNPs and one previously reported SNP were found by alignment of PCR products of Jinhua and Pietrain pigs. Based on the porcine *SIM1* cDNA sequence and human SIM1 genomic DNA sequence (GenBank accession no. NC_000006), two pairs of primers were designed to amplify the novel mutations (Table 1). The PCR program included: denaturation at 94°C for 5 min followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for SIM11 or 58°C for SIM12 for 30 sec and extension at 72°C for 30 sec with a final extension at 72°C for 10 min. All amplicons were subjected to PCR-single-strand conformational polymorphism (PCR-SSCP) analysis according to Zhao *et al*. (2008). The samples showing anomalous migration in polyacrylamide gel were sequenced to identify the SNP mutation type.

Statistical analysis: For studying the association between *SIM1* gene polymorphisms and recorded traits in the PJF2 crossbred animals were analyzed using GLM procedure (SPSS v16.0), the following model was used:

$$Y_{ijklm} = \mu + S_i + F_j + G_k + l_l + \beta * X_{ijklm} + e_{ijklm}$$

Where:

Y_{ijklm} = The observation of the trait
μ = Population mean
S_i = The fixed effect of sex
F_j = The fixed effect of father
G_k = The fixed effect of kth genotype
l_l = The random effect of litter
β = The regression coefficient of the slaughter weight
X_{ijklm} = The slaughter weight as covariate
e_{ijklm} = The random residual error

The model for intramuscular fat content trait include the average backfat thickness as covariate instead of slaughter weight. The Least Square Means (LSM) method of the GLM (General Linear Model) procedure was used for statistical analysis.

RESULTS AND DISCUSSION

Cloning of porcine *SIM1* gene: The 3720 bp porcine SIM1 cDNA sequence was obtained by splicing sequence of RT-PCR and RACE products further analysis revealed an Open Reading Frame (ORF) of 2301 bp which encoding a corresponding protein of 766 amino acid residues with a predicted molecular weight 85.3 kDa and theoretical isolectric point of 7.29. The cDNA contained a 5' Untranslated Region (UTR) of 461 bp nucleotides and a 3' UTR of 958 bp nucleotides. 3' UTR included a TGA termination codon and one putative polyadenylation consensus signals (AATAAA) and a poly (A) tail. The obtained cDNA and amino acids sequence were then submitted to the GenBank database (GenBank, Acc. nos. GU451304 and NP_001166056, respectively).

Expression of porcine *SIM1* gene in tissues: Semi-quantitative RT-PCR was used to detect expression profile of porcine *SIM1* gene. The electrophoresis results showed that the fragments of *SIM1* gene (176 bp) and β-actin gene (116 bp) were obtained (Fig. 1). The β-actin gene displayed a basically identical signals in all tissues. Porcine *SIM1* gene showed ubiquitous distribution in all examined tissues including stomach, spleen, pancreas, heart, lung, kidney, subcutaneous fat, muscle, liver and hypothalamus while particularly higher in kidney and muscle (Fig. 1).

Molecular phylogenetic tree: The alignment of nucleotide sequence showed that porcine SIM1 shared a high

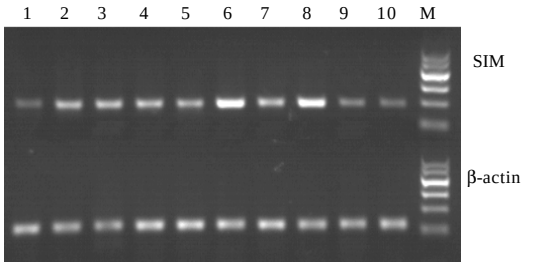

Fig. 1: Tissue expression distribution of porcine *SIM1* gene. 1: stomach; 2, spleen; 3, pancreas; 4, heart; 5, lung; 6, kidney; 7, subcutaneous fat; 8, muscle; 9, liver; 10, hypothalamus; M, DL600 markers, 6 bands: 100, 200, 300, 400, 500, 600 bp

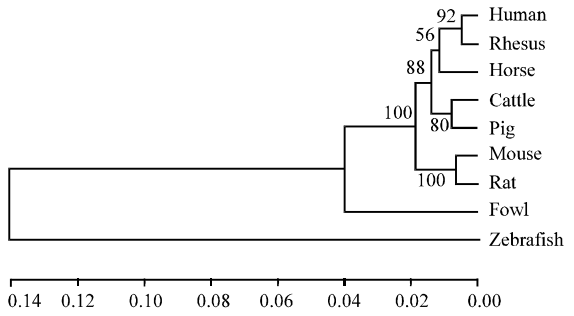

Fig. 2: The molecular phylogenetic tree of the *SIM1* gene conducted by UPGMAM algorithm of MEGA 3.1, bootstrap confidence values are based on 1000 bootstrap replicates. GenBank accession numbers are: human (NP_005059), rhesus (XP_001086054), horse (XP_001503954), cattle (XP_869704), pig (NP_001166056), Mouse (NP_035506), rat (NP_001101111), fowl (XP_419817), zebrafish (NP_835740)

identity with human (97.9%), cattle (96.7%), horse (97.1%), mouse (95.2%), rat (94.2%), monkey (97.7%), chicken (91.7%) and zebrafish (85.2%).

For functional domains, porcine SIM1 had high identity with nucleotide sequence of zebrafish in basic, HLH, PAS1 and PAS2 regions (92, 97, 95 and 98%, separately) while completely conserved sequences were observed in the four functional domains within porcine SIM1 and the other 7 species.

The amino acid sequence of porcine SIM1 showed high homologous to that of human and cattle (98.04 and 98.30%, respectively). A phylogenetic tree based on alignments of full-length amino acid sequence from nine species showed that the ortholog of porcine SIM1 is related to cattle counterpart and mammalian SIM1 are closely related to chicken SIM1 and zebrafish SIM1 (Fig. 2).

Detection of SNP: Alignment between Jinhua and Pietrain cDNA sequence revealed three potential Single Nucleotide Polymorphisms (SNPs). The first substitution located at position 534 (c+534 T>C) relative to the start codon, the second substitution located at position 990 (c+990 C>T) and the third one at position 1677 (c+1677 C>T). All of the three SNPs were silent mutations. The c+534 T>C and c+1677 C>T substitutions were novel SNPs, while the c+990 C>T was previously published by Zhao *et al.* (2008). The c+990 C>T substitution was excluded from genotype and association analysis in the present study as its research had been done in the same population by Zhao *et al.* (2008). Using PCR-SSCP and sequencing technology, two novel SNPs were genotyped in PJF2 population.

Association analysis: The association analysis between SNPs and recorded traits revealed that the *SIM1* gene had significant effect on carcass composition and meat quality. The substitution of c+534 T>C showed a extremely significant effect on average backfat thickness, intramuscular fat content, intramuscular water content and ham lean weight ($p<0.01$) furthermore, it was significantly associated with ham fat weight ($p<0.05$). Allele T in this site was associated with higher fat proportion and lower ham lean production (Table 2). The substitution of c.+1677 C>T had extremely significant effect on average backfat thickness, ham temperature and ham fat weight ($p<0.01$), significant effect was observed on loin eye area, ham pH and ham lean weight ($p<0.05$). Allele T in this site significantly decrease fat content but increase ham temperature, ham lean weight and loin eye area (Table 3). Both of the two SNPs have significant effect on average backfat thickness, ham fat weight and ham lean weight.

In the present study, we cloned and characterized the full cDNA length of porcine *SIM1* gene. Alignment analysis indicated that the *SIM1* gene has high homology to other species, especially in functional domains. The phylogenetic tree displayed that the porcine SIM1 protein has closely evolutionary relationship with mammals, the ortholog of porcine *SIM1* gene is related to its cattle counterpart. Therefore, cloning of porcine *SIM1* gene could enrich SIM1 family and afford suitable model for SIM1-related human disease. In human and mouse, *SIM1* gene expressed in the central nervous system as well as in other limited tissues such as kidney, lung and skeletal muscle (Chen *et al*., 1995; Ema *et al*., 1996). However in the present study, we found that the porcine *SIM1* gene had a wide distribution *in vivo* and expressed in 10 different tissues. Taking into account the previous studies focused on embryos but the study chose adult tissues as template thus, we speculated that SIM1 has

Table 2: Association analysis between c+534 C>T genotypes and recorded traits

Traits	Genotypes (Least square means±standard deviation) CC (n = 83)	CT (n = 118)	TT (n = 105)	p value
Average backfat thickness (cm)	2.830±0.058^{B}	2.996±0.049^{B}	3.221±0.050^{A}	0.000
Intramuscular fat content (%)	2.346±0.152^{B}	2.686±0.126^{B}	3.256±0.125^{A}	0.000
Intramuscular protein content (%)	24.244±0.123	24.238±0.105	24.153±0.101	0.779
Intramuscular water content (%)	73.688±0.178^{a}	73.403±0.152^{a}	72.872±0.146^{b}	0.001
Water holding capacity	0.407±0.013	0.413±0.011	0.440±0.011	0.104
Loin eye area (cm^2)	33.263±0.743	32.637±0.567	31.543±0.645	0.216
Ham temperature (°C)	39.339±0.150	39.267±0.126	39.301±0.129	0.928
Ham pH	6.340±0.039	6.347±0.033	6.372±0.033	0.782
Ham fat weight (kg)	1.912±0.037^{b}	1.967±0.032	2.036±0.032^{a}	0.043
Ham lean weight (kg)	5.726±0.056^{Aa}	5.618±0.047^{a}	5.482±0.049^{Bb}	0.004

LSM with superscripts a and b are different at $p<0.05$; A and B at $p<0.01$

Table 3: Association analysis between c+1677 T>C genotypes and recorded traits

Traits	Genotypes (Least square means±standard deviation) CC (n = 103)	CT (n = 141)	TT (n = 62)	p value
Average backfat thickness (cm)	3.145±0.052^{a}	3.032±0.045^{a}	2.848±0.065^{b}	0.001
Intramuscular fat content (%)	2.694±0.136	2.908±0.117	2.819±0.174	0.454
Intramuscular protein content (%)	24.337±0.107	24.103±0.093	24.226±0.135	0.219
Intramuscular water content (%)	73.143±0.159	73.315±0.139	73.374±0.202	0.572
Water holding capacity	0.424±0.110	0.424±0.100	0.407±0.015	0.560
Loin eye area (cm^2)	31.163±0.607^{b}	33.076±0.514^{a}	33.379±0.855^{a}	0.030
Ham temperature (°C)	39.231±0.132	39.255±0.114	39.514±0.166	0.008
Ham pH	6.324±0.034	6.399±0.029^{a}	6.300±0.043^{b}	0.075
Ham fat weight (kg)	2.052±0.033^{a}	1.982±0.028^{a}	1.839±0.042^{b}	0.000
Ham lean weight (kg)	5.505±0.050^{b}	5.611±0.043	5.728±0.064^{a}	0.016

LSM with superscripts a and b are different at $p<0.05$

temporal and spatial differences in gene expression. In human, Traurig *et al*. (2009) had identified 46 mutations in genomic DNA by sequencing and found a noncoding variant showing strong association with Body Mass Index (BMI) in Pima Indians. Although, none of the common SNP -IVS4+83GA, P352T, T361I, A371V, A665H- had significant association with BMI in different population (Ahituv *et al*., 2007; Traurig *et al*., 2009). The haplotype reconstructed of P352T and A371V was modestly associated with BMI in Caucasian males (Hung *et al*., 2007).

Overexpression of SIM1 in agouti yellow (A^y) mice inhibited hyperphagia and reduced fat mass in the A^y/SIM1 transgenic mice (Kublaoui *et al*., 2006), SIM1$^{-/-}$ and SIM1$^{+/-}$ mice developed severely obese with increased food intake and a remarkable decrease in hypothalamic Oxytocin (Oxt) and melanocortin 4 receptor (Mc4r) mRNA (Tolson *et al*., 2010). The SIM1$^{+/-}$ mice, however do not show decreased energy expenditure (Michaud *et al*., 2001). Based on these finding, it was proposed that *SIM1* gene may involved in the leptin-melanocortin-oxytocin pathway for hyperphagic obesity (Traurig *et al*., 2009; Tolson *et al*., 2010).

The two SNPs in the present study do not change primary structure of *SIM1* gene, but consecutive evidence demonstrated that synonymous mutation could affect SIM1 function by altering mRNA stem-loop structure or stability (Duan *et al*., 2003; Capon *et al*., 2004; Chamary and Hurst, 2005). In addition, *SIM1* gene was assigned at pig chromosome 1p13 (Zhao *et al*., 2008), this chromosome overlaps with several QTL affecting backfat thickness and total fat weight (Nezer *et al*., 2002; Geldermann *et al*., 2003).

The SIM1 protein contains four functional domains, including the basic, HLH and PAS (1 and 2) regions (Huang *et al*., 1993; Yamaki *et al*., 1996). The basic region mediate DNA binding while the HLH and PAS domains together direct the formation of protein dimmers and contributes to the stabilization of the DNA binding conformation (Huang *et al*., 1993; Erbel *et al*., 2003). In the present study, completely conserved sequences in SIM1 functional domains were observed among all species except zebrafish, this result was consistent with previous studies (Ema *et al*., 1996; Eaton and Glasgow, 2006). The similarity in nucleotide sequence represents a high potential for binding the same DNA sequence *in vivo* (Woods and Whitelaw, 2002) thus, it was expected that SIM1 proteins had conserved function within different species.

In relation to carcass traits, the average backfat thickness, ham fat weight, ham lean weight were significantly affected by both SNPs and loin eye area was significantly affected by T1677C substitution. Regarding the meat quality traits, intramuscular water content, ham temperature and ham pH were significantly affected by C534T and T1677C, separately.

CONCLUSION

In this study, *SIM1* gene had significant effect on porcine carcass composition and meat quality. *SIM1* gene is a candidate gene for economic traits. This result correspond with earlier inference (Zhao *et al*., 2008).

However, the association observed may arise from linkage disequilibrium within other regions of *SIM1* gene or causative substitution in neighbouring locus. Thus, further studies should be carried out in different populations and using larger population size to demonstrate the real impact of the *SIM1* gene on carcass and meat quality traits.

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