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Polymorphism and association analysis with carcass traits of porcine *USF1* gene

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ABSTRACT

The upstream stimulatory factor 1 (*USF1*) is a transcription factor controlling expression of several genes involved in lipid and glucose homeostasis. In this study, 2 isoforms of the porcine *USF1* gene were detected by reverse transcription polymerase chain reaction (RT-PCR), termed *USF1* wild-type (wt) and *USF1/CD*, both of them contain a helix-loop-helix leucine zipper (HLH-LZ) conserved domain. Tissue distribution analysis showed that the 2 transcripts of porcine *USF1* gene were ubiquitously expressed in all tested tissues, except for heart. Moreover, we found that a single nucleotide polymorphism (SNP, C/T) in intron 10 was significantly associated with ratio of lean to fat, dress percentage, average backfat thickness, loin eye width, lean meat percentage, loin eye height and loin eye area. This result suggested that porcine *USF1* gene might be a candidate gene of meat production trait.

Key words: Association analysis, Porcine, SNP, *USF1*

Upstream stimulatory factor 1 (*USF1*) is a member of the basic helix-loop-helix leucine zipper family of transcription factors (Gregor *et al.* 1990). It can form homodimers or heterodimers with *USF2*, which recognize E-box regulatory sequences and lead to transcription activation and/or enhanced gene expression (Sirito *et al.* 1992). The *USF1* gene located on human chromosome 1q22-q23 (Shieh *et al.* 1993) encodes a ubiquitously expressed transcription factor with multiple roles in transcriptional regulation of several genes involved in glucose and lipid metabolism (Lee *et al.* 2006). Polymorphisms of the *USF1* gene associated with familial combined hyperlipidemia (FCHL) (Pajukanta *et al.* 2004) increased risk for cardiovascular disease in women (Komulainen *et al.* 2006), high plasma triglyceride and low-density lipoprotein (LDL) levels (Coon *et al.* 2005), increased adipocyte lipolysis (Hoffstedt *et al.* 2005), type 2 diabetes (Meex *et al.* 2008) and diabetic kidney disease (Sanchez *et al.* 2011). *USF1* also represses the gene encoding ATP-binding cassette A1 (ABCA1) transporter protein, which has a key role in the cellular efflux of cholesterol and phospholipids (Yang *et al.* 2002). Additionally, *USF1* modulates genes involved in the immune response and cell cycle control (Corre and Galibert 2005, Terragni *et al.* 2011). Taken together, these genetic and biochemical data suggested that *USF1* may play an important role in regulating metabolic traits.

Hitherto, all studies about *USF1* have been carried out in mice and human, relatively little is known concerning the porcine *USF1* gene. In this study, we cloned porcine *USF1* cDNA and its partial genomic sequence, detected its polymorphism and performed an association analysis in a Yorkshire×Meishan pig cross. We also analyzed its tissue expression pattern. These will contribute to the further investigation on the physiological function of porcine *USF1* gene.

MATERIALS AND METHODS

Animals and samples: Eleven different tissues including heart, liver, spleen, lung, kidney, stomach, small intestine, ovary, uterus, longissimus dorsi muscle and backfat tissues were collected from a 4-month-old female Yorkshire pig, and then immediately frozen in liquid nitrogen and stored at –80°C for spatial expression analysis.

DNA extraction and cDNA synthesis: Genomic DNA was isolated according to the standard phenol–chloroform method and stored at –20°C. The RNA was extracted using Trizol reagent according to the manufacturer's protocol, treated with RNase-free DNase I to remove contaminating genomic DNA, incubate at 80°C with 2.5 µl of 0.5M EDTA for 2 min to inactivate DNase I and stored at –80°C. The first strand cDNAs was synthesized (Qiao *et al.* 2010), the corresponding cDNA was stored at –20°C until use.

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cDNA and genomic DNA cloning of porcine USF1 gene: The human *USF1* gene sequence (GenBank accession no. NM_007122) was applied to search available ESTs in porcine ESTs database with BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). According to the assembled contig sequences, 3 overlapping primer pairs U1F/U1R, U2F/U2R and U3F/U3R (Table 1) were designed to amplify the cDNA sequence of porcine *USF1* gene. Six primer pairs (U4F/U4R, U5F/U5R, U6F/U6R, U7F/U7R, U8F/U8R and U9F/U9R) (Table 1) were designed to amplify introns of the porcine *USF1* gene according to the above cDNA sequences were obtained. PCR reactions were performed in a total volume of 25 µl containing 1 µl (50 ng) of cDNA or DNA as template, 0.5 µl of each primer (5 µM), 2.0 µl of each dNTP (2 mM), 2.5 µl of 10×PCR buffer, 2.0 µl of (25 mmol/l) Mg^{2+} and 1µl (1 U/µl) of *Taq* DNA polymerase and 15.5 µl sterile water. The PCR amplification profiles were as follows: 94°C initial denaturation for 4 min, 35 cycles of 94°C denaturation for 40 s, annealing temperature for 40 s, and 72°C extension for 40–120 s (according the length of the target fragments), followed by a 10 min extension at 72°C. The PCR products were separated by 1.5% agarose gel electrophoresis, and purified using a gel extraction kit. The purified products were subcloned into the pMD–18T vector and sequenced by a commercial Company.

Bioinformatic analysis: We used NCBI's online ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to predict open reading frames for translated peptide products. The physico-chemical parameters of the deduced protein sequence were analyzed using ProtParam (http://cn.expasy.org/tools/protparam.html). Bioinformatics domain searching analysis was performed using PROSITE (http://au.expasy.org/prosite/). Multiple sequence alignments were carried out using the CLUSTALW 1.83 program (http://www.ebi.ac.uk/clustalw/) and the unrooted phylogenetic tree was constructed by DNAStar's LaserGene software. The 3–D structure of the putative protein conserved domain was analyzed using the 3-D Conserved Domain Architecture Retrieval Tool of Blast (http://www.ncbi.nlm.nih.gov/blast/).

Tissue expression analysis of the porcine USF1 gene: The specific primer pair U1F/U1R (Table 1) was designed to detect the expression of porcine *USF1* by semi-quantitative RT-PCR. House-keeping gene glyceraldehydes–3-phosphate dehydrogenase (*GAPDH*) which amplification spans an intron was used as an internal control to prevent DNA contamination. The PCR reaction was carried out with the following cycling parameters: 95°C initial denaturation for 4 min, 28 cycles of 95°C denaturation for 40 sec, 58°C annealing for 40 sec, and 72°C extension for 50 sec. A final extension was performed at 72°C for 7 min.

SNP identification, allele frequencies and association analyses: After compared the obtained sequences from Yorkshire and Chinese Meishan pigs, a C130T mutation was found in intron 10. Primer pair U9F/U9R was designed to distinguish the SNP well. The PCR restriction fragment length polymorphism (PCR-RFLP) method was used to genotype the polymorphic sites. A total of 142 DNA samples from unrelated animals representing four breeds (Yorkshire, Landrace and Chinese indigenous pig breed Meishan, Tongcheng) were genotyped and allele frequencies were determined. The population used in the association analysis consisted of an F_2 population involving Yorkshire and Meishan cross pigs (Zuo *et al.* 2005). All F_2 pigs were given twice daily diets formulated according to age under a standardized feeding regimen and free access to water. The F_2 pigs were slaughtered in 2003 and 2004 following a common protocol (Xiong and Deng 1999).

Table 1. Primers used in this study

Primer name	Primer sequence (52 to 32)	Annealing temperature (°C)	Product size (bp)
U1F	GTGAGGCAGGAGATACGAA	58	613 and 430
U1R	GGTCCTGGGAGCAATAGA		
U2F	GCGCTCTATTGCTCCCAG	57	312
U2R	CGAAGCACATCATTATCCAAC		
U3F	CAGGGGCTTGACCAACTG	62	520
U3R	TGCTTGTCAGCAGACCCT		
U4F	TCCAGGGAAATAGGAGCC	57	2094
U4R	CCTTCTTTGGAAGTCTTCGTAT		
U5F	GTGAGGCAGGAGATACGAA	55	740
U5R	GAAGACGTACTTGACGTTGG		
U6F	TGACCCCAACGTCAAGTA	60	783
U6R	TGCAGTGCTGGGGAAGTA		
U7F	ACCACATCGGGGAGTACA	59	600
U7R	AGCCCTGCGTTTCTCATC		
U8F	ACTCGGGATGAGAAACGC	59	656
U8R	CGAAGCACATCATTATCCAAC		
U9F	AGGGGCTTGACCAACTGC	62	690
U9R	TGCTTGTCAGCAGACCCT		
GA-PDHF	ACCACAGTCCATGCCATCAC	58	480
GAP-DHR	TCCACCACCCTGTTGCTGTA		

Statistical analysis: The association between different genotypes and carcass traits was performed with the least-squares method. Both additive and dominance effects were estimated using the REG procedure. The additive effect was defined as —1, 0 and 1 for CC, CT and TT, respectively, and the dominance effect represented as 1, —1 and 1 for CC, CT and TT, respectively. The statistical model was assumed to be: $Y_{ijk}=\mu+S_i+Y_j+G_k+b_{ijk}X_{ijk}+e_{ijk}$, where Y_{ijk} is the observed values of traits; µ is the least-square mean; S_i is effect of sex (I,1 for male or 2 for female), Y_j is the effect of year (j, 1 for year 2003 or 2 for year 2004), G_k is the effect of genotype (k, CC, CT and TT), b_{ijk} is the regression coefficient of the slaughter weight, X_{ijk} is the slaughter weight, and e_{ijk} is the random residual.

RESULTS AND DISCUSSION

Cloning and sequence analysis of the porcine USF1 gene: The cDNA sequences amplified by primer pairs U1F/U1R, U2F/U2R and U3F/U3R were 613 bp/430 bp, 312 bp and 520 bp, respectively. After assembling the sequences of RT-PCR products, 2 different transcript variants of porcine *USF1* were detected and designated as *USF1* (wt) and *USF1/CD*. The cDNA of *USF1* (wt) was 1382 bp, including an open reading frame (ORF) of 954 bp encoding a polypeptide of 317 amino acids with an estimated molecular mass of 34.3 kDa and an estimated isoelectric point of 5.26. Compared with *USF1* (wt), *USF1/CD* lacks the exon 6. The cDNA of *USF1/CD* was 1199 bp, including an open reading frame (ORF) of 771 bp encoding a polypeptide of 256 amino acids with an estimated molecular mass of 28.5 kDa and an estimated isoelectric point of 6.24. These sequence data were submitted to the GenBank database under GenBank accession no. EF 219407 for *USF1* (wt) and GenBank accession no. EF 208923 for *USF1/CD*.

A multiple alignment of deduced amino acid sequences of *USF1* (wt) is shown in Fig. 1. The predicted porcine *USF1* (wt) amino acid sequence had 97% identity with that of human, macaca and cow, 95% identity with that of mouse and rat, 86% identity with that of gallus. The porcine *USF1*

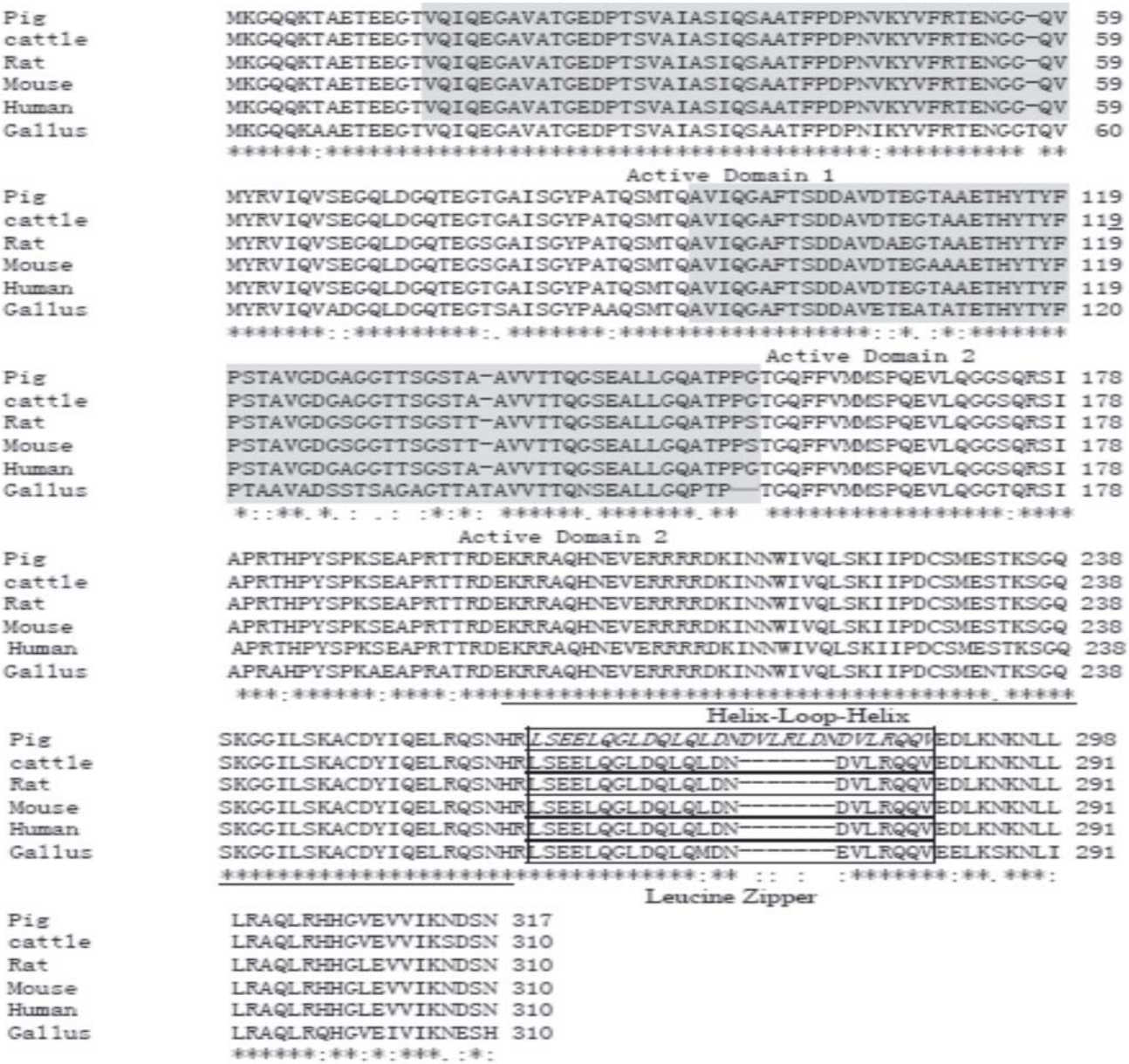

Fig. 1. Multiple amino acid sequence alignments of *USF1* gene. Protein sequences were obtained from GenBank: Cattle (NP_001001161), rat (NP_113965), mouse (NP_033506), gallus (NP_001007486), human (NP_009053) and our deduced amino acid sequence of porcine *USF1* (wt) (EF 219407). The grey part indicates active domain 1 and active domain 2. The helix-loop-helix domain is underlined. The leuine zipper is boxed. The symbols (*), (:) and (.) represented completely identical, conservative and semi-conservative amino acid residues respectively.

gene shares the high sequence identity with its mammalian counterparts at the amino acid level, which suggested the conservation of their biological functions during evolution. The predicted porcine *USF1* (wt) amino acid sequence contains 2 active domains, a helix-loop-helix motif and a leucine repeat. The leucine repeat of *USF1* was required for efficient DNA binding and *USF1* dimerization (Gregor *et al.* 1990). Compared with *USF1* (wt), *USF1/CD* lacks the exon 6, and lost the second activation domain. Transcriptional regulation seems to work in different ways. When some factors possessing the DNA-binding domains without the activation domains, heterodimerize with activators, either their DNA-binding affinity or their ability to activate transcription is reduced (Foulkes and Sassone-Corsi 2992). For example, a splice variant of human *USF1* gene, *USF1/BD*, lacks the first activation domain, but retains the DNA binding activity as a homodimer and a heterodimer with *USF1* (wt), and acts as a modulator for *USF1* (Saito *et al.* 2003). In this study, the porcine *USF1/CD* has the first trans-activation domain and bHLH domain of *USF1*, suggesting that *USF1/CD* may play a role as a modulator of *USF1* to control the expression of target genes.

The overlapping primer pairs U1F/U1R–U9F/U9R (Table 1) were designed to amplify the genomic sequence of the porcine *USF1* gene. The amplified 5516 bp sequence was deposited into GenBank with GenBank accession no. EF 625884. In accordance with human orthologous, sequence analysis revealed that porcine *USF1* (wt) gene consists of 11 exons and 10 introns and the locations of splice donor/acceptor sites in all introns conform to the "GT/AG" rule (Table 2).

Phylogenetic analysis of USF1: In an effort to determine the evolutionary relationship between the different *USF1* proteins, a phylogenetic tree was generated. Six different *USF1* proteins were used for the phylogenetic analysis. These can be seen to separate into different clades, which reflect their evolution (Fig. 2). The results revealed that the porcine *USF1* had the closest genetic relationship with the *USF1* of Cattle. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

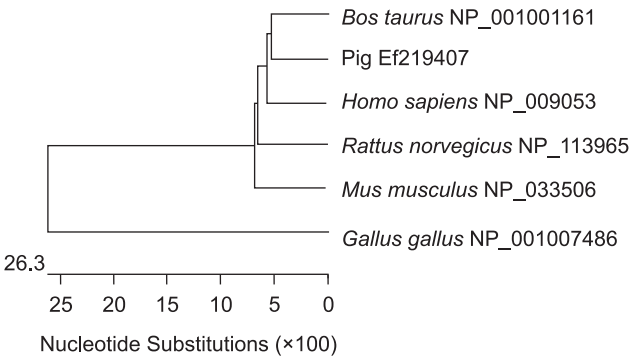

Fig. 2. The *USF1* phylogenetic tree with amino acid sequences. The sequences used for analysis are derived from GenBank, and their accession numbers are shown on the *right hand side*. The horizontal branch lengths are proportional to the estimated divergence of the sequence from the branch point.

Tissue expression pattern of porcine USF1 (wt) and USF1/CD: USF1 (wt) displayed a significantly high expression in kidney, followed by spleen, backfat, lung, uterus, ovary, stomach and longissimus dorsi muscle, and extremely low in small intestine and liver, but no expression was detected in heart (Fig. 3). *USF1/CD* was expressed in all tissues examined with abundant transcript in kidney. These showed that the 2 different transcripts may have distinct functions in heart tissue. The tissue expression patterns of genes can somehow imply their corresponding functions. The high expression of *USF1* in kidney suggested

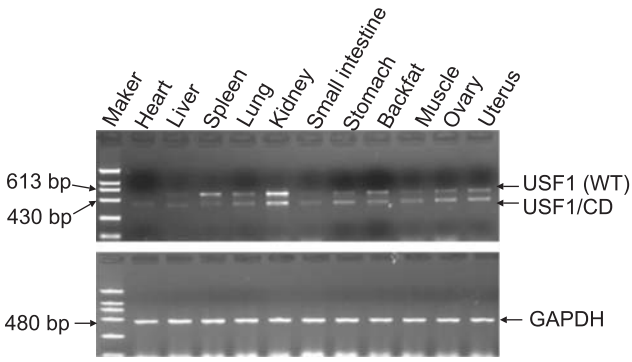

Fig. 3. Expression of porcine *USF1* (wt) and *USF1/CD* gene in different tissues in Yorkshire by RT-PCR. Marker, DL2000.

Table 2. Exon-intron junctions of the porcine *USF1* gene

Exon	Exon size (bp)	5′ Splice donor	Intron	Intron size (bp)	3′ Splice acceptor
1	35	TGGCTG/gtgagt	1	2007	taccag/GACTTA
2	127	GAAGGG/gtgagt	2	363	aaacag/GCAGCA
3	50	ATTCAG/gtgcgg	3	157	attcag/GTGCGG
4	116	GGCCAG/gtaagg	4	301	ctctag/GTGATG
5	102	ACCCAG/gtacag	5	241	tcccag/GCCGTG
6	196	GCACTG/gtgaga	6	249	tcacag/GTCAGT
7	88	TTCCCC/gtgagt	7	135	tgttag/GAAGTC
8	59	ATGAAG/gtgggt	8	153	ccacag/TGGAAC
9	95	GGCCAG/gtcatg	9	249	ttacag/AGTAAA
10	150	CAGCAG/gttaga	10	192	tgtcag/GTGGAA
11	90				

Table 3. Alleles frequencies of the polymorphic site located within intron 10 in different pig breeds

Breed	Number of pigs	Genotype frequencies			Allele frequencies	
		CC	CT	TT	C	T
Yorkshire	37	37	0	0	1	0
Landrace	43	35	3	5	0.8488	0.1512
Meishan	39	7	2	30	0.2051	0.7949
Tongcheng	23	3	2	18	0.1739	0.8261

that the gene might have important functions related to diabetic kidney disease (Sanchez *et al*. 2011). The expression in backfat indicated that it may be related to lipid metabolism. The expression in ovary and uterus implied that the gene might have important functions related to reproductive capacity of pigs. To explain these tissue expression differences explicitly, further research based on these primary results is needed.

Polymorphism detection and association analysis: Sequence comparisons between Yorkshire and Meishan pigs revealed 1 SNP (C130T) in intron 10 of the porcine *USF1* gene. The SNP could be detected by PCR-*Alu*I-RFLP, three genotypes were presented as: CC (413 bp and 277 bp), TT (413 bp, 176 bp and 101 bp) and CT (413 bp, 277 bp, 176 bp and 101 bp) (Fig. 4). We analyzed this polymorphism in 142 unrelated pigs including indigenous (Meishan and Tongcheng) and foreign breeds (Yorkshire and Landrace) by means of the PCR-RFLP using primer pair U9F/U9R (Table 1). Genetic variation analysis revealed that allele frequencies were significantly different between Chinese indigenous breeds and western commercial pig breeds. The Chinese indigenous breeds had higher frequencies of the T allele (Table 3).

The investigation on the effect of the C130T polymorphism on carcass traits in the Yorkshire × Meishan F_2 population and the experimental populations, showed that the porcine *USF1 Alu*I polymorphism had significant associations with ratio of lean to fat ($P < 0.05$), dress percentage ($P < 0.05$), average backfat thickness ($P < 0.05$), loin eye width ($P < 0.05$), lean meat percentage ($P < 0.01$), loin eye height ($P < 0.01$) and loin eye area ($P < 0.01$). The lean meat percentage, ratio of lean to fat, loin eye height, loin eye width and loin eye area of pigs with CC genotype were significantly higher than those of pigs with TT genotype, and the average backfat thickness of pigs with CC genotype was significantly thinner than that of pigs with TT genotype (Table 4).

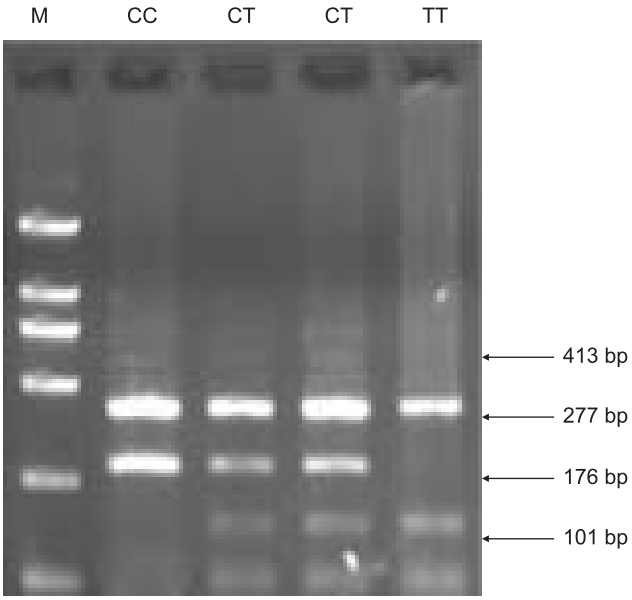

Fig. 4. PCR-*Alu*I-RFLP analysis of porcine *USF1* gene. Lane M is marker DL2000. Lane CC, CT and TT represent different genotypes.

The porcine *USF1* gene was mapped to SSC4q (Moller *et al*. 2004). SSC4q harbours QTLs associated with fatness, growth and carcass traits (Andersson *et al*. 1994, Knott *et al*. 1998). Our statistical analysis also indicated that the polymorphic site in this gene had a significant correlation

Table 4**.** Association analysis of porcine *USF1* genotypes with carcass traits

Traits	Genotype (μ±SE)			Genetic effect (μ±SE)	
0	CC (n = 88)	CT (n = 208)	TT(n = 125)	Additive	Dominance
LMP(!)	55.380±0.460^{A}	56.138±0.357^{B}	54.595±0.343^{A}	–0.355±0.292	–0.568±0.233**
RLP	2.579±0.089^{a}	2.762±0.069^{b}	2.569±0.066^{a}	0.006±0.058	–0.092±0.046*
DP	71.441±0.595^{a}	72.026±0.460^{b}	72.855±0.442^{b}	0.689±0.374*	0.044±0.298
ABT	2.448±0.045^{a}	2.491±0.047^{ab}	2.508±0.061^{b}	0.046±0.027*	0.017±0.030
LEH	8.472±0.094^{A}	8.365±0.130^{B}	8.401±0.168^{B}	–0.399±0.155**	–0.208±0.123*
LEW	4.779±0.108^{a}	4.761±0.140^{a}	4.696±0.078^{b}	–0.314±0.132**	0.107±0.105
LEA	31.295±0.397^{A}	30.765±0.414^{A}	29.295±0.533^{B}	–0.746±0.332*	–0.614±0.265*

LMP, lean meat percentage; RLF, ratio of lean to fat; DP, dress percentage; ABT, average backfat thickness; LEH, loin eye height; LEW, loin eye width; LEA, loin eye area.Significant levels between the genotype classes a, b, and an asterisk (*) indicated significant difference at Pdþ0.05 level; A, B, and a double asterisk (**) indicates significant difference at Pdþ0.01 level.

with carcass traits. Allele T seemed to be associated with increase of fat deposition traits such as average backfat thickness, but decrease of traits related to muscle traits such as lean meat percentage and loin eye area. Therefore, allele C presented positive and desirable effects on muscle growth traits, but negative on the fat deposition traits. As allele C was predominant in western commercial pigs and present as the low frequency allele in Chinese indigenous pigs, the effects of allele were also consistent with the breed differences. Therefore, porcine *USF1* gene may be an important candidate gene of growth and carcass traits and the association results in our study indicated that the SNP may simply be used as genetic markers linking to quantitative trait loci with effects on carcass traits.

In conclusion, we have isolated and characterized the porcine *USF1*. Data presented here provide biochemical and structural bases for future studies on porcine *USF1* function and will potentially lead to a better understanding of porcine *USF1*.

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