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Isolation, Expression Pattern, Polymorphism and Association Analysis of Porcine TIAF1 Gene*

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ABSTRACT : TIAF1 is a TGF-β1-induced anti-apoptotic factor that plays a critical role in blocking TNF (tumor necrosis factor) cytotoxicity in mouse fibroblasts and participates in TGF-β-mediated growth regulation. In this study, we obtained the full-length cDNA sequence of the porcine *TIAF1* gene. Real-time PCR further revealed that the *TIAF1* gene was expressed at the highest level in liver and kidney with prominent expressions detected in uterus, and lower levels detected in heart, spleen, lung, stomach, small intestine, skeletal muscle and fat of Large White pigs. Sequence analysis indicated that a 6 base-pair deletion mutation existed in the exon of the *TIAF1* gene between Meishan and Large White pigs. This mutation induced deletion of Gln and Val amino acids. PCR-RFLP was used to detect the polymorphism in 394 pigs of a "Large White×Meishan" F_2 resource population and four purebred pig populations. The frequencies of the A allele (with a 6 bp deletion) were dominant in Chinese Meishan and Bamei pigs, and the frequencies of the B allele (no 6 bp deletion) were dominant in Large White and Landrace pigs. Association analyses revealed that the deletion mutation had highly significant associations ($p<0.01$) with meat marbling score of the thorax-waist *longissimus dorsi* (LD) muscle (MM1) and intramuscular fat percentage (IMF), and significant associations ($p<0.05$) with carcass length (CL). The results presented here supply evidence that the 6 bp deletion mutation in the *TIAF1* gene affects porcine meat quality and provides useful information for further porcine breeding. (**Key Words :** *TIAF1*, Porcine, Real-time PCR, Carcass Traits, Meat Quality Traits, Deletion Mutation)

INTRODUCTION

Some studies have indicated that there is significant phenotypic variance between Chinese indigenous pigs and Western pigs. Meishan pigs (Chinese indigenous pigs) have lower lean meat content in their carcasses compared to Large White pigs (Western pigs), but the lean meat of Meishan pigs is of better quality (Serra et al., 1992). And it is well documented that Meishan pigs have super reproduction traits (Chen et al., 2008). Liver is the most important organ in the process of substrate metabolism by organisms. Glycometabolism, lipid and protein metabolism occur in the liver. Phenotypic variance between Large White and Meishan pigs may be related to substrate metabolism which occurs in the liver. We have identified a set of genes differentially expressed in livers between these two breeds by the mRNA differential display technique (DDRT-PCR) which was first reported by Liang and Pardee (1992) as a fast and efficient method for investigating differences in gene expression.

Among those differentially expressed genes we identified up-regulation of the *TIAF1* (TGF-β1-induced anti-apoptotic factor 1) gene transcripts in Meishan pigs compared with Large White pigs. Human *TIAF1* gene is located on chromosome 17q11.2 and encodes a protein of 115 amino acids (http://www.ncbi.nlm.nih.gov/). *TIAF1* plays a critical role in blocking TNF (tumor necrosis factor) cytotoxicity in mouse fibroblasts (Chang et al., 1998; van der Leij et al., 2003). The *TIAF1* expression is regulated by TGF-β1 (transforming growth factor-beta 1) which is a pleiotropic cytokine with demonstrable effects on a wide variety of cellular targets (Bonewald, 1999) and its principal role is as an immune regulator (Shull et al., 1992). Smad2, Smad3, and Smad4 were used as receptors and intracellular signal-transducing proteins by TGF-β to regulate extracellular matrix homeostasis, apoptosis, cell

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Table 1. Primers used in this study

Primers	Primer sequences (5′-3′)	*Tm* (°C)	Product size (bp)	Template
EST167F	CACCCTCAAGGTCATCCCAC	58	124	cDNA
EST167R	CACAGCCCAATGAGTACAAAC			
Ct1F	TCAGATGATGAGCACGACC	58.5	771	cDNA
Ct1R	AACCCAAACCCAGAAGCA			
Ct2F	AGAGGTGGAGGGAAGAGC	58.5	1,015	cDNA
Ct2R	AGCACAGCCCAATGAGTA			
5′RACE	AACGCAGAGTACGCGGG	56	415	cDNA
Ct3R	TGGGCTAGGCGTTGGTCT			
Ct4F	GGACATTCTCGGGTTGCT	58	601	DNA
Ct4R	TCCATCCTCAGGCTTTCT			
Ct5F	GCCTACTGGGACATTCTCG	60	483	DNA
Ct5R	GTGGGTGGGATGGGTTTA			
RtF	TGAGCAAACCTCCACAGAC	58	179	cDNA
RtR	CCAGGCAACTTGGACAGAG			
GAPDHF	ACCACAGTCCATGCCATCAC	58	452	cDNA
GAPDHR	TCCACCACCCTGTTGCTGTA			
β-actinF	CCAGGTCATCACCATCGG	58	158	cDNA
β-actinR	CCGTGTTGGCGTAGAGGT			

proliferation, and others (Lawrence, 1996; ten Dijke et al., 2000). Khera and Chang (2003) reported that TIAF1 participates in TGF-β-mediated growth regulation. All these studies indicated that the *TIAF1* gene might play an important role in apoptosis and cell proliferation of animals. However, there is no report on the function of *TIAF1* in pigs.

This study aimed to investigate the functional importance of the porcine *TIAF1* gene by analyzing its sequence characteristics and spatial expression profiles. Association analysis between the deletion mutation and carcass and meat quality traits was performed also in 394 pigs of the "Large White×Meishan" F_2 resource population.

MATERIALS AND METHODS

Experimental animals

In this study, all pigs came from the Jingpin pig station of Huazhong Agricultural University. Screening for polymorphisms in the porcine *TIAF1* gene was performed in a total of 160 pigs of Large White (n = 30), Landrace (n = 44), Bamei (n = 46) and Meishan (n = 40) breeds. For association analysis, carcass and meat quality traits were recorded in 394 pigs of a "Large White×Meishan" F_2 resource population. The Meishan pig (Chinese indigenous pig) is a typical lard type breed and the Large White pig (Western pig) is a lean type breed. All the F_2 pigs were fed as described by Dai et al. (2006). The finishing animals were slaughtered in the years 2000 (including 51 dams and 49 sires which were produced by mating 5 males to 16 females), 2003 (including 79 dams and 71 sires which were produced by mating 5 males to 22 females) and 2004 (including 73 dams and 71 sires which were produced by mating 5 males to 22 females), and measured according to the method of Xiong and Deng (1999) in the Center of Swine Control of China (Wuhan). All experimental procedures were approved by Hubei Province Committee on Laboratory Animal Care. Genomic DNA was isolated from blood samples of all 554 pigs using a standard phenol: phenol/chloroform purification-based protocol.

cDNA synthesis and differential display of mRNA

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from livers of 12 two-month old pigs (six Meishan pigs and six Large White pigs) according to the manufacturer's instructions and treated with DNase I (Promega, Madison, USA) to exclude the DNA contamination. All the harvested samples were treated as described by Gong et al. (2008). Total RNA from the six Meishan pigs and six Large White pigs was mixed to construct the RNA pools. The first strand cDNA was synthesized from 2 μg total RNA in a 25 μl reaction system containing 0.5 μg oligo$(dT)_{15}$ primer, 1×M-MLV first-strand buffer, 200 U M-MLV reverse transcriptase (Promega), 12.5 mM dNTP and 25 U RNasin Ribonuclease Inhibitor (Promega).

Differential display PCR was carried out simultaneously with 15 arbitrary primers in combination with 10 anchor primers. PCR amplification was performed as described by Liu et al. (2004). The PCR products were separated on an 8% non-denaturing polyacrylamide gel (29:1). The bands differentially displayed were re-amplified, cloned, and sequenced, and then the obtained sequences were compared with those available in GenBank using Blast search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Semi-quantitative reverse transcription–polymerase chain reaction

The primer pair EST167F/EST167R (Table 1) was

designed to identify the differentially displayed band, and a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as described by Ren et al. (2006). The house-keeping gene glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) (Table 1) was also amplified as internal control.

cDNA isolation, sequencing and analysis

The differentially expressed cDNA fragment (EST167) was homologous to the human *TIAF1* gene with 81% identity. So the cDNA sequence of the human *TIAF1* gene (GenBank accession No. NM_004740) was used to search available ESTs in the 'EST-others' database with the standard BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The porcine ESTs which shared more than 80% homology with the human cDNA sequence were assembled to produce an EST-contig. In order to obtain the full-length cDNA sequence of the porcine *TIAF1* gene, primer pairs Ct1F/Ct1R and Ct2F/Ct2R were designed according to the contig (Table 1), and 5' rapid amplification of cDNA ends (RACE) was used according to BD SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, USA) with the primer pair 5' RACE/Ct3R (Table 1).

Polymorphism detection and association analysis

To screen polymorphisms, the primer pair Ct4F/Ct4R was designed based on the PCR fragment which covered the complete coding regions of the *TIAF1* gene. Potential polymorphic sites were analyzed by sequence comparison using DNAstar software (DNAstar Inc., Madison, WI, USA). The primer pair Ct5F/Ct5R was used to amplify the fragment from genomic DNA to genotype the polymorphic site in the *TIAF1* gene. The association between genotypes and carcass and meat quality traits was evaluated by the least squares method (GLM procedure, SAS version 8.0, 1999). According to the method of Liu (1998), both additive and dominance effect were also estimated using the REG procedure of SAS version 8.0, where the additive effect was denoted as -1, 0 and 1 for AA, AB and BB, respectively, and the dominance effect was represented as 1, -1 and 1 for AA, AB and BB, respectively. The model used to analyze the data was assumed to be:

$$Y_{ijkl} = \mu + S_i + Y_j + G_k + B_l + bX_{ijkl} + e_{ijkl}$$

where Y_{ijkl} is the observation of the trait; μ is the population mean, S_i is the effect of i-th sex (i = 1 for male or 0 for female), Y_j is the effect of j-th year (j = 1 for 2000, 2 for 2003 or 3 for 2004), G_k is the effect of k-th genotype (k = AA, AB or BB), B_l is the effect of l-th boar, b is the regression coefficient of the slaughter weight (carcass traits) or the regression coefficient of the slaughter age (meat quality traits) and e_{ijkl} is the random residue.

PCR reaction amplification was performed in 25 μl reaction volume on a GeneAmp PCR system 9600 (Perkin Elmer, Foster City, CA, USA). Mixes comprised 100 ng porcine genomic DNA or cDNA as template, 0.5 μM of each primer, 200 μM of each dNTP, 1×PCR buffer (with NH_4^+), 1.5 mM of $MgCl_2$ and 1 U *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). The PCR amplifications were performed with the following cycling parameters: initial denaturalization at 94°C for 4 min, 35 cycles of 94°C for 45 s, annealing at optimal temperature (Table 1) for 45 s, and 72°C for 1 min. A final extension was performed at 72°C for 10 min. PCR products were gel purified, cloned to vector pMD18-T (Takara, Dalian, China) and sequenced commercially.

Real-time PCR analysis of porcine TIAF1 expression levels

Total RNA was extracted from 10 tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, uterus, skeletal muscle and fat) of 3 two-month old Large White pigs. Reverse transcriptase PCR was performed as described above. The primer pair RtF/RtR (Table 1) was used to determine the relative expressed status of the *TIAF1* gene with real-time PCR. The reaction mixes comprised 0.5 μl porcine cDNA, 0.25 μM each primer, and 12.5 μl SYBR Green Real-time PCR Master Mix (TOYOBO, Tokyo, Japan). The PCR amplifications were performed in 25 μl with an iCycler 5 iQ real-time PCR system (Bio-Rad). PCR conditions for the expression profiles were as follows: 95°C for 1 min, then followed by 40 cycles (95°C for 15 s, 58°C for 15 s, and 72°C for 20 s). A threshold cycle (Ct) value for each sample was calculated by the levels of fluorescence. The relative expression levels of the gene were analyzed using the Comparative Ct method, in which the house-keeping gene *β-actin* (Table 1) was used as an internal control, to correct for the differences in the mRNA quantities.

RESULTS

Identification of EST167 as differentially expressed gene between Meishan and Large White pigs by DDRT-PCR

A band, designated as EST167, which expressed higher in the liver of Meishan pigs but lower in Large White pigs, was isolated from the mRNA differential display gel (Figure 1(A)). Analysis of semi-quantitative RT-PCR also found that EST167 presented higher expression in the liver of Meishan pigs, but lower in Large White pigs. This was approximately consistent with the result of mRNA differential display (Figure 1(B) and (C)).

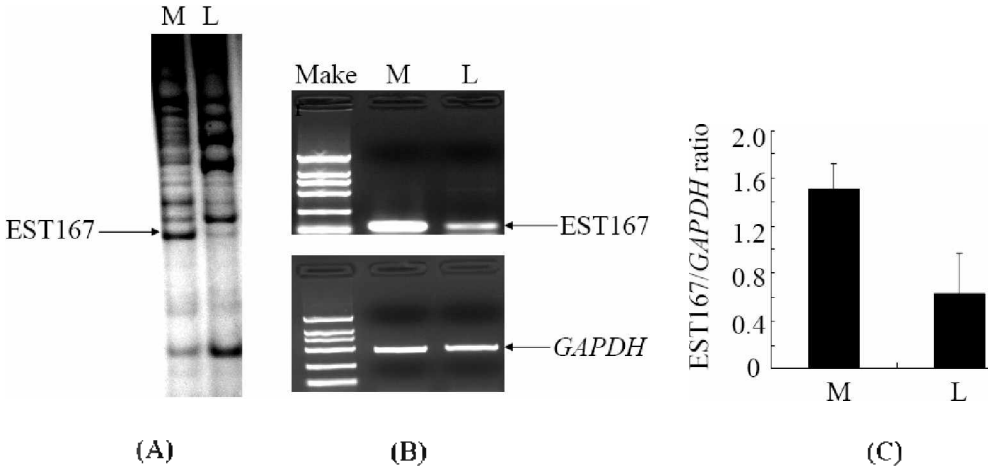

Figure 1. The results of differential display and identification of EST167 as differentially expressed gene in the liver between Meishan and Large White pigs by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). M and L represent Meishan and Large White pigs, respectively. (A) The arrow points to EST167. (B) Semi-quantitative RT-PCR analysis of EST167. Marker, Marker DL2000 DNA Ladder (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp) (TaKaRa). (C) The bar graph of the ratio of EST167/*GAPDH*; Bars represent the mean±SE (n = 3).

Molecular cloning and sequence analysis of porcine TIAF1 gene

The full-length cDNA sequence of the porcine *TIAF1* gene was cloned and assembled to obtain 1,633 bp (GenBank accession No. EU872207) including a complete open reading fragment (ORF) of 348 bp encoding a protein of 115 amino acids, 905 bp of 5' UTR and 380 bp of 3' UTR containing a consensus polyadenylation signal (AATAAA). Alignment analysis revealed that the porcine *TIAF1* gene has only one exon. Similarity comparison showed that the nucleotide sequences of porcine *TIAF1* shared, respectively, 79% and 81% identity with that of human (GenBank accession No. NM_004740) and mouse (GenBank accession No. AF104984).

Polymorphism detection and allele frequencies

The primer pair Ct4F/Ct4R amplified a total of 601 bp of the porcine *TIAF1* gene from four different individuals (two Large White pigs and two Meishan pigs). Comparison of those sequences revealed a 6 base-pair deletion mutation from 1,001 bp to 1,006 bp in the exon (GenBank accession No. EU872207) which caused Gln and Val amino acid deletion; further analyses of the deletion mutation were carried out using the restriction fragment length polymorphism (RFLP) method by the restriction enzyme *Eco47 I*. The primer pair Ct5F/Ct5R was used to distinguish the genotype. PCR products which had a 6-bp deletion mutation could not restrict with the enzyme *Eco47 I* and were designated as A allele (477 bp), and those which did not have the deletion mutation can restrict with the enzyme *Eco47 I* and were designated as B allele (304 bp and 179 bp) (Figure 2). We next performed statistical analysis and found that the frequencies of the A allele were dominant in Chinese indigenous breeds and the frequencies of the B allele were dominant in Western pig breeds (Table 2). In order to estimate the association between this polymorphism and carcass and meat quality traits, *Eco47 I*-PCR-RFLP was typed in 394 pigs of the "Large White× Meishan" F_2 resource population. Association analysis revealed that the deletion mutation had highly significant associations ($p<0.01$) with meat marbling score of the thorax-waist *longissimus dorsi* (LD) muscle (MM1) and

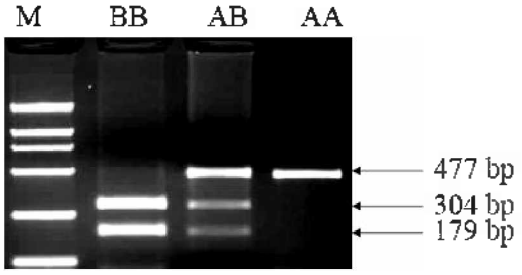

Figure 2. Restriction pattern of the amplification with the primer pair Ct5F/Ct5R. Agarose gel electrophoresis (2.0%) showing polymorphisms in PCR fragments of the *TIAF1* gene after digestion with *Eco47 I*. The genotypes are shown at the top of the lanes, M, Marker DL2000 DNA Ladder (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp) (TaKaRa).

Table 2. Allele frequencies for the deletion mutation of the *TIAF1* gene in four pig populations

Breeds	Number	Genotype			Allele frequency	
		AA	AB	BB	A	B
Bamei	46	30	11	5	0.772	0.228
Meishan	40	35	3	2	0.913	0.087
Landrace	44	0	0	44	0	1
Large White	30	2	11	17	0.250	0.750

Table 3. Association between porcine *TIAF1 Eco47 I*-PCR-RFLP and carcass and meat quality traits in 394 pigs of the "Large White×Meishan" F2 resource population

Traits[1]	Genotype (Lsmean±SE)[2]			Effect (mean±SE)	
	AA (n = 221)	AB (n = 105)	BB (n = 68)	Additive	Dominance
CL (cm)	92.095±0.457[a]	90.250±0.545[b]	90.347±0.659[b]	0.874±0.614	-0.971±0.509
MM1	3.507±0.017[A]	3.431±0.021[B]	3.383±0.028[B]	0.063±0.021**	-0.014±0.018
IMF (%)	3.424±0.050[Aa]	3.303±0.065[a]	3.050±0.083[Bb]	0.188±0.048**	0.066±0.040

[1] CL = Carcass length; MM1 = Meat marbling score of the thorax-waist *longissimus dorsi* (LD) muscle; IMF = Intramuscular fat percentage.

[2] Least square mean values (±SE).

[3] Different letters denoting significant difference between groups: [a, b] $p<0.05$, [A, B]** $p<0.01$. n, the number of individuals.

intramuscular fat percentage (IMF), and significant associations ($p<0.05$) with carcass length (CL). At this locus, the additive effect seemed to be highly significant ($p<0.01$) and allele A was associated with increases of the trait value (Table 3).

Spatial expression pattern of the TIAF1 gene

The spatial expression analysis showed that the *TIAF1* gene was expressed at the highest level in liver and kidney with prominent expressions detected in uterus, and lower levels detected in heart, spleen, lung, stomach, small intestine, skeletal muscle and fat of Large White pigs (Figure 3).

DISCUSSION

In this study, we selected the differentially expressed cDNA fragment (EST167) which was homologous to the human *TIAF1* gene for future studies according to the important function of this gene, and isolated porcine *TIAF1* gene fragments with corresponding knowledge of the known human *TIAF1* gene by bioinformatics methods. This proved to be a rapid and effective method to obtain genomic sequence of the candidate genes (Wang et al., 2007).

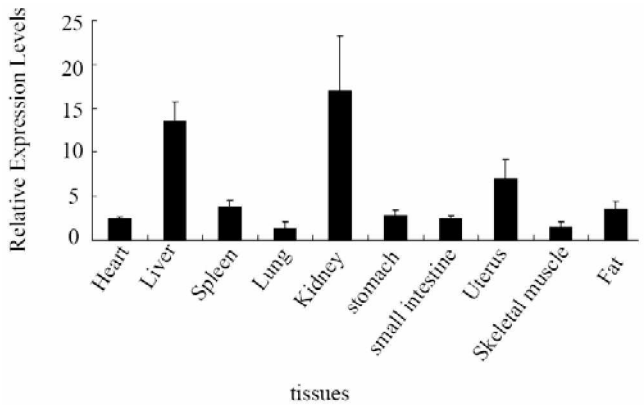

Figure 3. Spatial expression profile of the *TIAF1* gene in different tissues of two-month old Large White pigs by real-time PCR. Relative expression levels of this gene were analyzed using the Comparative Ct method, employing *β-actin* as the reference gene in each sample. Bars represent the mean±SE (n = 3).

GenBank BLAST analysis revealed that the *TIAF1* gene was not homologous to any of the known porcine genes, so the gene was a novel porcine gene.

We used the real-time PCR technique to detect the tissue-specific expression of these mRNAs in pigs. The spatial expression analysis showed that the *TIAF1* gene was widely expressed in all the examined tissues of Large White pigs. This result is similar to the study of Chang et al. (1998) which showed that the *TIAF1* gene is ubiquitously expressed in a variety of tissues and organs, such as liver, eyes, stomach, intestine, heart, lung, spleen, testis, ovary, pituitary gland, skeletal muscle and pregnant uterus. These results indicated that TIAF1 might play important roles in various tissues and organs.

TIAF1 protects L929 fibroblasts from TNF-mediated apoptosis, but over-expressed TIAF1 induces growth inhibition and apoptosis of monocytic U937 and non-fibroblast cells (Chang, 2004; Schultz et al, 2004). TIAF1 participates in the TGF-beta signaling by interacting with Smad proteins (Chang, 2003), and the TGF-betas are potent modulators of cell proliferation, cell differentiation, and fibrogenesis (Hellerbrand et al., 1999). TGFβ1 and TNFα (tumor necrosis factor alpha) can stimulate the transdifferentiation of fat-storing cells into highly active and "synthetic" myofibroblast–like cells in the rat liver (Bachem et al., 1993). The deletion mutation of porcine *TIAF1* gene, which was found in its exon, causes deletion of two amino acids, which might alter the protein structure and lead to functional variation such as the interaction with TGFβ1. The changes may affect fat metabolism and synthesis, which may be the reason the polymorphism had highly significant association with fat depositional traits. According to the association result, if A frequency were increased meat marbling score of the thorax-waist LD muscles, intramuscular fat percentage and carcass length could be greatly improved. Increasing allele A may be favorable for carcass and meat quality traits in pig breeding. Analysis of allelic frequency also revealed great difference between lean- and lard-type breeds. Chinese indigenous breeds (Meishan and Bamei) have higher frequencies of allele A, whereas Large White and Landrace pigs have higher frequencies of allele B. This may be caused by the limited number of animals or long-term and different

background of breeding and selection. If the polymorphism effect was the same as found in our study for other larger populations, it may be a useful marker for further porcine breeding.

In this study, we first isolated the full-length cDNA sequence of the *TIAF1* gene, analyzed its spatial expression differences, found an important functional deletion mutation, and analyzed the significant association with carcass and meat quality traits in pigs. In summary, this information provides an important basis for conducting future studies on the function of the *TIAF1* gene.

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