



Transcriptional analysis of the porcine *TTID* gene and association of different *TTID* genotypes with carcass traits

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ABSTRACT. The titin immunoglobulin domain (*TTID*) protein localizes to the Z line in muscle and binds to alpha-actinin and gamma-filamin. It plays an indispensable role in stabilizing and anchoring of thin filaments. In this study, the 5'-regulatory region of the porcine *TTID* gene was analyzed with bioinformatic methods. Another objective of this study was to further investigate the polymorphism in the intron 6 of the porcine *TTID* gene. We determined allele frequency among six Chinese porcine purebreds. The polymorphisms were genotyped in a population of 280 F₂ pigs representing two Large White x Meishan reference families. Different *TTID* genotypes were significantly associated with carcass traits, including skin percentage ($P < 0.05$), loin eye area ($P < 0.05$), and average skin thickness ($P < 0.01$). Our study will continue to lay

the groundwork for further investigations into the detailed function of the porcine *TTID* gene.

Key words: Porcine; Titin immunoglobulin domain protein (TTID); Polymerase chain reaction-restriction fragment length polymorphism; Association analysis; Carcass traits

INTRODUCTION

Sarcomeres are the basic contractile units of the striated muscle, which is composed of thin actin and thick myosin filaments. The sarcomere is controlled by many structural proteins, such as titin, nebulin and others (McElhinny et al., 2005). Titin is a giant protein that extends to one and a half of the length of the sarcomere; the titin N-terminus is embedded within the Z-disk and its C-terminus within the M-line (Gautel et al., 1999). The titin protein contains several immunoglobulin (Ig)-like domains and some unstructured peptide sequences (Salmikangas et al., 1999). Human titin immunoglobulin domain (TTID) protein was the first titin protein studied: it is a novel 57-kD cytoskeletal protein that co-localizes and directly interacts with alpha-actinin in sarcomeric I-bands (Salmikangas et al., 1999). The human *TTID* gene mapped to the human chromosome 5q31, between markers AFM350yB1 and D5S500; it contains 10 exons that extend over 19 kb (Salmikangas et al., 1999; Godley et al., 1999). Studies have shown that TTID plays an indispensable role in stabilizing and anchoring of thin filaments, which may be a prerequisite for correct Z-disc organization (Salmikangas et al., 2003). In porcine, Davoli et al. (2002) only mapped the gene to SSC 2q26.

Our previous study has shown that porcine *TTID* mRNA is expressed at the highest level in skeletal muscle and at the second-highest level in the heart (Wang et al., 2013), consistent with findings of *TTID* expression in human tissues (Salmikangas, 2001). In addition, a T978C single nucleotide polymorphism (SNP) in intron 6 of porcine *TTID* gene has been identified by *HinfI* PCR-RFLP. The distribution of the allele frequency in four different pig breeds has been analyzed by Wang et al. (2013), indicating that the Chinese breed Meishan have a higher frequency of the A allele (77.4%), whereas Large White and Landrace pigs display higher frequencies of the B allele (100 and 98.2%, respectively). Association analysis showed that some T978C SNP genotypes were significantly associated with meat pH (musculus biceps femoris) ($P < 0.05$), meat color value (musculus biceps femoris) ($P < 0.05$), or water moisture (musculus biceps femoris) ($P < 0.05$) (Wang et al., 2013).

The current study was designed to further analyze the deduced protein sequence of porcine TTID and to investigate possible effects of the T978C polymorphism in porcine *TTID* intron 6 on carcass traits. This study will contribute additional knowledge of the function of the porcine *TTID* gene.

MATERIAL AND METHODS

The TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and TESS (<http://www.cbil.upenn.edu/cgi-bin/tess>) programs were used to analyze putative transcriptional factor-binding sites in the nucleotide sequence of the 5'-flanking region of the porcine *TTID* gene (GenBank accession No. DQ157551).

Two F₂ cross-breeding population between Large White and Meishan pigs were generated in 2000 and 2003, respectively, and consisted of 280 animals (Yang et al., 2010). All animals had unlimited access to food and water and were born and raised in Huazhong Agriculture University Jingpin Pig Station. Pigs were slaughtered when they were 180 days old and measured according to the methods of Xiong and Deng (1999). The measured carcass traits were the following: birth weight, dressing percentage, skin percentage, bone percentage, internal fat rate, fat meat percentage, lean meat percentage, ratio of lean meat to fat meat, carcass length, rib numbers, loin eye height, loin eye area, average skin thickness, backfat thickness at shoulder, backfat thickness at thorax-waist, backfat thickness at buttock, backfat thickness at 6-7th thorax, and average backfat thickness (measured at three points: backfat thickness at shoulder, backfat thickness at thorax-waist, backfat thickness at buttock).

Blood samples were collected from 280 F₂ individuals. Genomic DNA was isolated by phenol/chloroform purification protocols (Sambrook et al., 1989) and stored at -20°C.

The intron 6 of porcine *TTID* gene was amplified by PCR with the primers Genomic-F (5'-CTGCTCCCAATTCAGAAAA-3') and Genomic-R (5'-GGATGAGTGATGATTTGTTTG-3'). PCR amplification was carried out in a 25-μL volume containing standard 1X PCR buffer and 1 IU Taq polymerase (Jingmei Biotech, China), 200 μM of each dNTP, 10 pmol of each primer, and 1.0 μL genomic DNA. The template was denatured for 4 min at 94°C, followed by 35 cycles of amplification at 94°C for 50 s, 59°C for 60 s, 72°C for 50 s, and terminated with an additional extension step for 10 min at 72°C. A fragment of 1402 bp was amplified in this way, and three genotypes of T978C SNP were identified as AA (44, 519, and 839 bp), AB (44, 519, 838, 411, and 428 bp), and BB (44, 519, 411, and 428 bp) by PCR-*Hinf*I-RFLP as previously described (Wang et al., 2013).

The association between genotype and carcass and meat-quality traits was performed with a least-square method (GLM procedure, SAS 8.0). The additive and dominance effects were estimated using the REG procedure of SAS 8.0. The additive effect was defined as -1, 0, and 1 for AA, AB, and BB, respectively, and the dominance effect represented as 1, -1, and 1 for AA, AB and BB, respectively. The statistical model was assumed to be the following:

$$Y_{ijk} = \mu + S_i + F_j + G_k + b_{ijk}X_{ijk} + e_{ijk}$$

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 and 2 for female); F_j is the effect of family; G_k is the effect of genotype ($K = AA, AB$ and BB); b_{ijk} is the regression coefficient of the slaughter age; X_{ijk} is the slaughter age; e_{ijk} is the random residual (Liu, 1998).

RESULTS

Sequence analysis for the 5'-flanking region upstream of the transcriptional start site revealed putative binding motifs for several transcription factors: one site each for activating protein-1 (AP1), hairy and enhancer of split 1 (HES1), Yin Yang 1 (YY1), and a CACCC box; three sites each for CCAAT/enhancer-binding protein β (C/EBP β) and the SP1 transcription factor (Figure 1).

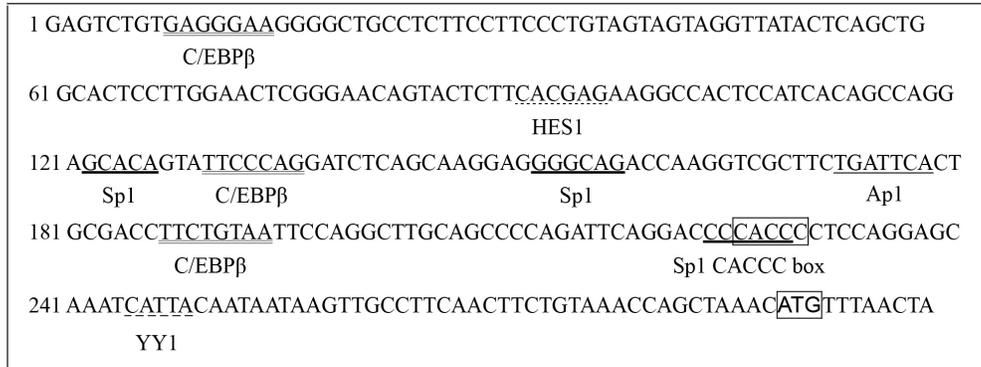


Figure 1. Nucleotide sequence of the 5'-flanking region of the porcine *TTID* gene. Potential transcriptional elements are underlined.

The result of the PCR amplification of the 1402-bp fragment is shown in Figure 2. The T978C polymorphisms were analyzed by PCR-*HinfI*-RFLP (see Figure 3).

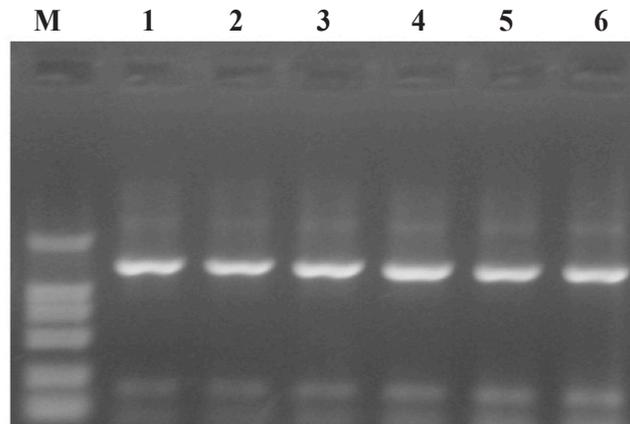


Figure 2. Amplified fragment of F₂ generation from the Large White x Meishan resource family; Lane M = DL2000 marker. Lane M = DNA DL2000 markers (100, 250, 500, 750, 1000, and 2000 bp); lanes 1-6 = amplified fragment of part samples from resource family, 1402 bp.

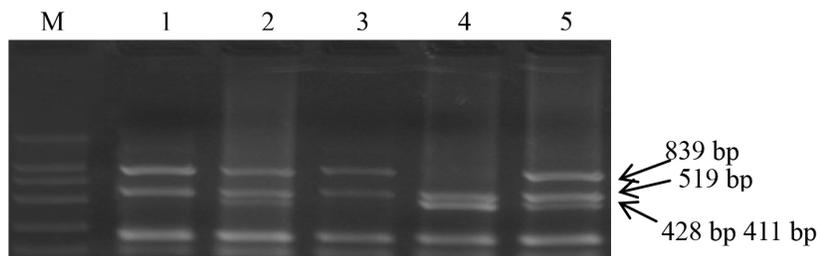


Figure 3. Agarose gel electrophoresis (1.5%) showing the *HinfI*-RFLP results of the *TTID* gene. Lane M = DNA marker DL2000; lanes 1, 3 = genotype AA, 839, 519, 44 bp; lanes 2, 5 = genotype AB, 839, 519, 428, 411, 44 bp; lane 4 = genotype BB, 519, 428, 411, 44 bp.

The allele distribution of the porcine *TTID* polymorphism in 87 unrelated pigs was analyzed. The results of this analysis indicated that all six porcine purebreds in this site were polymorphic at the *TTID* locus (Table 1). Four breeds (Yangxin, Qingping, Wannanhua, and Bamei pigs) comprised three genotypes (AA, AB and BB), 2 breeds (Erhualian and Jianli) had no genotype BB, which is roughly consistent with previous studies (Wang et al., 2013). The allele frequencies of this polymorphism displayed difference in the six breeds: Erhualian and Jianli pigs had 86.4 and 95% frequency, respectively, of the *A* allele, higher frequencies than were present for this allele in the other four breeds (Table 1).

Table 1. Distribution of *Hinf*I-RFLP genotype and allele frequencies among six pig breeds.

Breed	Number	Genotype AA	Genotype AB	Genotype BB	A allele frequency (%)	B allele frequency (%)
Erhualian	11	8	3	0	86.36	13.64
Jianli	20	18	2	0	95.00	5.00
Yangxin	13	6	5	2	65.38	34.62
Qingping	14	2	8	4	42.86	57.14
Wannanhua	18	5	12	1	61.11	38.89
Bamei	11	1	6	4	36.36	63.64

A total of 280 F₂ pigs of a Large White x Meishan resource population were used to identify polymorphisms by PCR-*Hinf*I-RFLP. Of this population, 49 were of genotype AA, 129 of AB, and 102 of BB, thus corresponding to relative genotype frequencies of AB > BB > AA in this resource population.

The mean weight of 280 F₂ pigs was 86.8 kg (SD = 8.3), and the association analysis results are listed in Table 2. As can be seen from Table 2, the *Hinf*I SNP was significantly associated with skin percentage (P < 0.05), loin eye area (P < 0.05), and average skin thickness (P < 0.01).

Table 2. Statistical analysis of *TTID* *Hinf*I-RFLP genotypes with carcass traits.

Traits	<i>TTID</i> <i>Hinf</i> I-RFLP genotype			Genetic effects	
	Genotype AA	Genotype AB	Genotype BB	Additive	Dominance
BWT	1.108 ± 0.054	1.109 ± 0.031	1.174 ± 0.034	0.040 ± 0.030	0.023 ± 0.021
DP	71.368 ± 0.696	72.326 ± 0.396	72.100 ± 0.434	0.231 ± 0.390	-0.216 ± 0.270
SP	10.466 ± 0.283 ^a	10.268 ± 0.161 ^{ab}	9.816 ± 0.177 ^b	-0.354 ± 0.159*	-0.134 ± 0.125
BP	13.315 ± 0.321	13.199 ± 0.183	12.782 ± 0.200	-0.301 ± 0.180	-0.134 ± 0.124
IFR	2.982 ± 0.115	3.119 ± 0.066	2.962 ± 0.072	-0.044 ± 0.065	-0.076 ± 0.045
FMP	22.250 ± 0.816	21.906 ± 0.464	21.996 ± 0.508	-0.077 ± 0.456	0.080 ± 0.316
LMP	53.865 ± 0.711	54.443 ± 0.405	55.233 ± 0.443	0.708 ± 0.398	0.203 ± 0.277
RLF	2.745 ± 0.162	2.721 ± 0.092	2.704 ± 0.101	-0.020 ± 0.091	-0.003 ± 0.063
CL	90.528 ± 0.625	91.422 ± 0.356	90.819 ± 0.390	-0.025 ± 0.351	-0.342 ± 0.242
RNS	14.540 ± 0.110	14.736 ± 0.063	14.754 ± 0.069	0.087 ± 0.062	-0.021 ± 0.043
LEH	6.689 ± 0.343	6.547 ± 0.195	7.005 ± 0.214	0.226 ± 0.192	0.184 ± 0.133
LEA	27.737 ± 0.727 ^a	28.934 ± 0.414 ^{ab}	29.682 ± 0.453 ^b	0.922 ± 0.407*	0.101 ± 0.284
AST	0.418 ± 0.016 ^A	0.392 ± 0.009 ^{AB}	0.369 ± 0.010 ^B	-0.024 ± 0.009**	-0.005 ± 0.006
BFT1	3.534 ± 0.130	3.571 ± 0.074	3.445 ± 0.081	-0.063 ± 0.073	-0.050 ± 0.050
BFT2	2.769 ± 0.097	2.783 ± 0.055	2.684 ± 0.060	-0.055 ± 0.054	-0.038 ± 0.038
BFT3	2.013 ± 0.087	2.041 ± 0.049	2.010 ± 0.054	-0.008 ± 0.049	-0.015 ± 0.034
BFT4	1.870 ± 0.104	1.837 ± 0.059	1.825 ± 0.065	-0.020 ± 0.058	0.000 ± 0.040
ABF	2.474 ± 0.095	2.467 ± 0.054	2.426 ± 0.059	-0.028 ± 0.053	-0.014 ± 0.037

Data are reported as means ± SE. *P < 0.05 for lowercase superscript letters. **P < 0.05 for capital superscript letters. BWT = birth weight; DP = dressing percentage; SP = skin percentage; BP = bone percentage; IFR = internal fat rate; FMP = fat meat percentage; LMP = lean meat percentage; RLF = ratio of lean meat to fat meat; CL = carcass length; RNS = rib numbers; LEH = loin eye height; LEA = loin eye area; AST = average skin thickness; BFT1 = backfat thickness at shoulder; BFT2 = backfat thickness at thorax-waist; BFT3 = backfat thickness at buttock; BFT4 = backfat thickness at 6-7th thorax; ABF = average backfat thickness.

The association of loin eye height with genotype AA versus genotype BB tended to be statistically significant ($P < 0.06$). This locus appeared to be significantly associated with additive effects on some carcass traits, such as skin percentage ($P < 0.05$), loin eye area ($P < 0.05$), and average skin thickness ($P < 0.01$).

DISCUSSION

As is well known, the sequence of a gene is an entry point into studying the gene's function (Wu et al., 2006). In this study, we analyzed the 5'-flanking region upstream of the transcriptional start site of the porcine *TTID* gene. Our analysis suggested that *TTID* expression is regulated by several transcription factors, including SP1, AP1, C/EBP β , and HES1. AP1 is known to be involved in regulating inflammatory responses. C/EBP β plays important roles in many cellular responses by regulating the transcription of genes in target cells, including genes involved in cell proliferation and differentiation, tumorigenesis and apoptosis, and cell cycle regulation (Ramji and Foka, 2002). YY1 activates and represses transcription of a large number of cellular and viral genes, and plays an important role in development and differentiation (Yao et al., 1998). In addition, the expression of the transcription factor SP1 is induced by hypoxia (Szalad et al., 2009), which may be adapted to the need of muscle hypoxia in the process of *TTID* gene activity. We also identified one site for HES1 and a single CACCC box in the putative *TTID* gene promoter. HES1 belongs to the basic helix-loop-helix (bHLH) family of transcription factors and is one of the seven members of the HES gene family (HES1-7), encoding nuclear proteins that suppress transcription and affect cell proliferation and differentiation in embryogenesis (Kageyama et al., 2007). CACCC (or GGGTG)-boxes and related GC-rich elements are present in the promoters and enhancers of a large number of mammalian and viral genes, and numerous transcription factors have been identified that bind to these elements to either activate or repress gene expression (van Vliet et al., 2000). Thus, we suggest that the expression of the *TTID* gene may be regulated at the transcription level, with a number of transcription factors contributing to its complex regulation.

Studies have shown that the porcine *TTID* gene is associated with the SSC2q24-q29 QTL marker (Davoli et al., 2002). The SSC2 region is considered to have some QTLs for average backfat, average daily gain, meat androstenone content, 16-day weight, drip loss, off-flavor, pH, and also other traits (Thomsen et al., 2004; Liu et al., 2007). It is well known that density and diameter of muscle fiber are negative correlated with meat quality (Gao et al., 2009). We chose the gene *TTID* as a candidate gene to study and analyze because its encoded protein is an important member of the titin protein family, playing an indispensable role in stabilizing and anchoring thin filaments by binding to alpha-actinin and gamma-filamin (Salmikangas et al., 1999; Wang et al., 2013).

Identifying SNPs within important functional regions of the candidate gene to enable analyses of the association of identified SNPs with important economic traits is a very useful tool to study gene function (Wang et al., 2005). In this study, we have continued to analyze the A T978C SNP in intron 6 of the porcine *TTID* gene on the basis of our previous research (Wang et al., 2013) by analyzing this polymorphism in six pig breeds indigenous to China. The results indicated some differences in allele frequencies and genotype distribution among these purebred pigs: two breeds (Erhualian and Jianli pigs) were identified as having higher *A* allele frequencies and two types of genotype distribution (AA and AB). The *A* or *B* allele frequencies in the other four breeds ranged from 34.6 to 65.4%, and three *TTID* genotypes were identified

in these four breeds. These results may have been affected by long-term breeding and high selection pressure or limited number of animals in this study.

In pig breeding, meat-quality and carcass traits are considered to be important economic characteristics (Wang et al., 2012). In this study, we analyzed the association between *TTID* gene polymorphisms and carcass traits in a 280-pig resource population. Association analysis showed that the skin percentage and loin eye area of pigs with an AA genotype were higher than those of pigs with a BB genotype ($P < 0.05$), and that the average skin thickness of pigs with an AA genotype were significantly higher than those of pigs with a BB genotype ($P < 0.01$). These results indicated that the SNP might be used as a simple genetic marker linked to QTL for predicting effects on carcass traits. Combined with results from a previous study investigating associations of gene polymorphisms with meat-quality traits (Wang et al., 2013), the B allele could be beneficial to accelerate the genetic improvement of pig breeds in terms of lean growth and meat quality. According to our results, the pig *TTID* gene is located close to the QTLs *SW240-SO226* and *SW2623-SO141*, affecting pH and drip, and *TTID* may be responsible for these two QTLs or other QTLs close to them. Therefore, we may use this site as a molecular marker that can be applied to marker assisted selection in pig breeding. However, the number of pigs analyzed was limited in this study, and other closely linked genes on *SSC2* might have affected our observations. Therefore, studies on more pigs of crossbreds and purebreds are needed to confirm these association results between the PCR-*HinfI*-RFLP and carcass traits in this study or meat-quality traits in the previous study by Wang et al. (2013).

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