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Characterization of the fast skeletal troponin C (*TNNC2*) gene in three Chinese native sheep breeds

Abstract

Fast skeletal muscle troponin C (*TNNC2*) protein plays a critical role in skeletal muscle contraction and has been proposed to be involved in meat quality traits in farm animals. Here, we report the cloning of the gene *TNNC2* from native Chinese sheep. Sequence analysis indicated that the gene *TNNC2* contains a putative open reading frame of 483 base pair, encoding a protein of 160 amino acids. We employed the polymerase chain reaction-single strand conformation polymorphism analysis to study the relationship between the *TNNC2* gene polymorphisms and meat quality traits. A single site mutation (T/−) was detected in the first intron, which created three genotypes TT (T/T), TD (T/−) and DD (−/−). Compared with the DD and TD genotypes, the genotype TT conferred lower in carcass weight ($P<0.05$), *longissimus thoracis et lumborum* shear force ($P<0.05$) and drip loss rate ($P<0.05$), and higher in pH value ($P<0.01$) and marbling score ($P<0.01$). The results indicated that the allele T had positive effects on tenderness and marbling score, and suggest that the allele could be used as a molecular marker to study the meat quality in sheep.

Keywords: sheep, cloning, *TNNC2*, meat quality, polymorphism

Zusammenfassung

Titel der Arbeit: **Charakterisierung des schnellen Muskelfaser Troponin C (*TNNC2*) Gens in drei einheimischen chinesischen Schafrassen**

Troponin C der schnellen Muskelfasern (TNNC2) spielt eine wichtige Rolle bei der Muskelkontraktion und der Ausprägung von Fleischqualitätsmerkmalen beim Nutztier. Hier wird über die Klonierung des *TNNC2* des Schafs berichtet. Die Sequenzanalyse weist ein offenes Leseraster von 483 Basenpaaren aus, die 160 Aminosäuren kodieren. PCR-SSCP (single strand conformation polymorphism) Analysen wurden durchgeführt, um den Zusammenhang zwischen *TBNNC2* Polymorphismen und Fleischqualitätsmerkmalen zu untersuchen. Im ersten Intron wurden eine Insertion/Deletion (T/–) identifiziert mit den entsprechenden Genotypen TT (T/T), TD (T/–) and DD (–/–). Der Genotyp TT ist gegenüber DD und TD assoziiert mit geringerem Schlachtgewicht ($P<0.05$), Scherkraft im *M. longissimus thoracis* und *lumborum* ($P<0.05$), Tropfsaftverlust ($P<0.05$), höherem pH-Wert ($P<0.01$) und Marmorierung ($P< 0.01$). Die Ergebnisse zeigen, dass das T Allel vorteilhaft hinsichtlich Zartheit und Marmorierung ist und als Marker für weitere Untersuchungen zur Fleischqualität beim Schaf genutzt werden könnte.

Schlüsselwörter: Schaf, Klonen, TNNC2, Fleischqualität, Polymorphismus

Introduction

For decades, consumers consider tenderness as the most important attribute of meat quality (THEIL et al., 2006; MULLEN et al., 2006). However, the genetic mechanism of tenderness has not been elucidated. Increasing tenderness in valuable tough meat by traditional selective breeding remains a difficult task, because the tenderness was controlled by minor-polygene (BENDIXEN, 2005) and its genetic underlying is complex. The marker-assisted selection based on investigating the associations of candidate gene polymorphism and traits has advantages for meat quality traits selection (WIMMERS et al., 2005).

Skeletal muscle tissue becomes meat after slaughtered in meat producing animals, skeletal muscle development and skeletal myoblast differentiation are very important for the amount of meat deposition in these animals. Meat tenderness associated with fibre types of muscle (KARLSSON et al., 1993; O'HALLORAN et al., 1997; WIMMERS et al., 2005), in which the fibre types can be classified according to their contractile nature (FONSECA et al., 2003). Thus, the genes involved in skeletal muscle development, myoblast differentiation or skeletal muscle contraction are considered usually as potential candidate genes for meat quality (TE PAS and SOUMILLION, 2001). As a subunit of Troponin C (TnC), fast skeletal troponin C (*TNNC2*) plays a critical role in skeletal muscle contraction (FARAH and REINACH, 1995). *TNNC2* gene is expressed during the myoblast differentiation and skeletal muscle development (BUCHER et al., 1988). Moreover, the intracellular Ca^{2+} concentration, an important factor affected the meat tenderness of pig (KÜCHENMEISTER and KUHN, 2003), is primary functional factor for *TNNC2* activity (YE et al., 2006). Hence, it is essential to test the association between *TNNC2* and meat quality. Previous studies about *TNNC2* gene mostly concerned on the effect of structure on muscle contraction in human and model animals (GILLIS and TIBBITS, 2000; RAMOS et al., 2004). The aim of this study is to investigate the effects of *TNNC2* single nucleotide polymorphism (SNP) on sheep meat quality traits in three Chinese native sheep populations by means of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP).

Materials and methods

Sheep populations

Sixty days old lambs (n=178) of three Chinese native sheep breeds were selected based on the criteria that there had non-pedigreed lambs within breed (rams : ewes=1 : 1). Three breeds were Small-Tailed Han sheep from Jining city of Shandong province (35.38° N, 111.6° E), Tan sheep from Helan county of Ningxia Hui Autonomous Region (38.55° N, 106.35° E) and Inner Mongolia sheep from Alasan League of Inner Mongolia Autonomous Region (38.85° N, 109.11° E). All lambs were maintained at the uniformed feeding conditions in the farm of Animal Science and Technology College, Northwest A & F University.

Meat quality traits and sampling

The lambs were slaughtered at 90 days old after fasting for 18 h with free access to water. Immediately after slaughter, the blood samples were collected (10 mL) in EDTA-coated tubes for subsequent DNA isolation. The *longissimus thoracis et lumborum* (LT) tissue samples (10 g) were fresh-frozen in liquid nitrogen then stored at −80 °C for reverse transcription (RT)-PCR.

Body weight and hot carcass weight were recorded at slaughter. Loin-eye area was determined by tracing the LT muscle surface area at the 10^{th} rib and by using a compensating polar planimeter. The fresh sample of between 4^{th} and 6^{th} lumbar was removed from left carcass, cut into small blocks (5×5cm) immediately for measuring the Warner-Bratzler shear force and meat colour according to the description of FIEMS et al. (2000). The pH value was measured by a pH-meter after slaughtered 45 min using a pH temperature probe inserted 2 cm at 8^{th} rib of carcass directly. Drip

loss rate was determined as the method described by HONIKEL (1998). Meat marbling scores were analyzed according to XING and DENG (1999)'s report.

TNNC2 gene cloning and sequencing

Using *Bos taurus TNNC2* complete coding sequence (GenBank accession no. BC 118491) in a BLAST search of *Ovis aries* expressed sequence tags (ESTs) database. A putative cDNA sequence of sheep *TNNC2* gene was assembled by the ESTs which have high identity (>85%) with the probe sequence. Based on the assembled sequence, the *TN1* primer (Table 1) was designed to amplify entire coding regions of the *TNNC2* gene. Total RNA were extracted from LT muscle samples with Trizol reagent according to the manufacture instruction (Invitrogen Inc., Carlsbad, CA). Two μg of total RNA were reverse-transcripted into cDNA with 50 nM oligo(dT)18 primer (TaKaRa, Dalian, China) and 200 U MMLV reverse transcriptase (Invitrogen Inc., Carlsbad, CA). PCR was carried out in a 25 μL reaction volume containing: 2 μL cDNA, 2.5 mM $MgCl_2$, 0.4 μM TN1F., 0.4 μM TN1R (Table 1), 0.4 mM dNTPs and 1.25 U DNA polymerase (TaKaRa, Dalian, China). The PCR started with denature at 94°C for 4 min, followed by 34 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 45 s), and ended with final extension at 72°C for 10 min.

A ~500 base pair (bp) DNA fragments purified from the gel were subcloned into the pMD18-T plasmid vector using the TA cloning kit (TaKaRa, Dalian, China). Positive clones were sequenced from both directions by the automated PE377 DNA sequencer (ShangHai Biotechnology CO, Ltd.) with M13 forward and reverse primers.

Table 1
Primer sequences of cloning and polymorphism analysis of *TNNC2* gene in three Chinese native sheep breeds (Primersequenzen für die Analyse von *TNNC2* in drei einheimischen chinesischen Schafrassen)

Primer name	Primer sequences[a]	Amplified regions[b]	Amplicon size and Tm
TN1	F: 5′–ATGACGGACCAGCAGGCTG–3′ R: 5′–CTTCTTACTGCACGCCCTC–3′	CDS	483bp, 58.0°C
TN2	F: 5′–ATTTGTGTGCAGGAGAACGG–3′ R: 5′–GCTCCTCTTTGGTGGGTG–3′	Exon 1, intron 1 and part of exon 2	399bp, 55.0°C
TN3	F: 5′–GGCCGCCTTTGACATGTTCG–3′ R: 5′–TCGAAGATGCGGAAACACTCAGC–3′	Part of exon 2, intron 2 and exon 3	323bp, 59.5°C

[a]F: forwards; R: reverse; [b]The amplified regions of *TN2* and *TN3* were overlapped in exon 2.

Polymorphisms analysis

Genomic DNA was extracted by phenol/chloroform purification method (SAMBROOK et al., 1989). To amplify exon1 to exon3 of *TNNC2*, the *TN2* and *TN3* primers were designed based on the sequenced results of the cDNA and *Bos taurus* genomic DNA sequence (Accession: NC_007311). The protocol of PCR reaction was same as that of abovementioned, except annealed at 55°C and 59.5°C for *TN2* and *TN3* primers, respectively.

Genotypes were detected according to following program: five μL PCR products were mixed with 7 μL loading dye (98% formamide, 10 mM EDTA, 0.025% bromorphenol blue and 0.025% xylene-cyanol) denatured for 10 min at 98°C, chilling on ice and loaded on polyacrylamide gel. Electrophoresis was performed by using protein II xi cells (Beijing Liuyi CO, Ltd.) at 150 V, 4°C for 14 h in 1×TBE buffer. Silver staining was as described by BASSAM et al. (1991). The PCR products showing different band patterns on SSCP gel were selected for sequence from both directions by the automated ABI377 DNA sequencer (ShangHai Biotechnology CO, Ltd.).

Statistics analysis

The associations between sheep LT meat quality traits and polymorphism was analyzed with 60 individuals of Small-Tailed Han sheep, 58 individuals of Tan sheep and 60 individuals of Inner Mongolia sheep, respectively.

Genotype distribution for Hardy-Weinberg equilibrium was tested by the method described by FALCONER and MACKAY (1996). The general linear model (GLM) procedure of SAS 8.0 (SAS Inst. Inc., Cary, NC 1999) was used to analyze the relationship between genotypes and meat quality traits by means of the least square method. Pearson's correlation coefficients among meat quality traits were obtained by PROC CORR of SAS.

The model used to analyze the data is assumed to be:

$$Y_{ijk}=\mu+S_i+B_j+G_k+(BG)_{jk}+E_{ijk}$$

where: Y_{ijk} = the observation value of the trait; μ = the population mean; S_i = the fixed effect of sex; B_j = the effect of j-th breed; G_k = the effect of k-th genotype and E_{ijk} = the random error. Additive effects and dominance effects were calculated separately. The effects of farm and age were not included into the linear model because all lambs at same ages were raised in the same farm from 60 to 90 days old.

Results

Cloning and sequence analysis

The strategies for amplified the sequence of sheep *TNNC2* gene were summarized in Figure 1. The resultant sequencing indicates that the full length cDNA of *TNNC2* is 483 bp and contains an open reading frame (Figure 1). The sequence was submitted to GenBank (GenBank accession no. EU239357).

Fig. 1: Nucleic acid sequence and predicted amino acid sequence of sheep *TNNC2* gene

The mRNA sequence containing the open reading frame was shown in uppercase letters; whereas the 5′– and 3′– untranslated regions are shown in lowercase letters. Positions of the start (ATG) condon and stop (TAA) codon are shaded. Primer sequences are in blocks. Polyadenylation signal is underlined. The amino acids sequence is shown in uppercase letters below the nucleic acid sequence. The sequence that shows in italic with lowercase letters is intron 1. The (t–) represents the SNP site, in which the base pair was "t" or deletion.

(Nuklein- und Aminosäurensequenz des ovinen *TNNC2*)

The formatted ClustralW alignment (Figure 2) revealed that sheep *TNNC2* protein shares the homologous with *Bos taurus* (GenBank accession no. NP_001069841), *Sus scrofa* (GenBank accession no. AAS88728), *Homo sapiens* (GenBank accession no. CAG46682), *Mus musculus* (GenBank accession no. NP_033420) and *Gallus gallus* (GenBank accession no. NP_990781), in which the sequence identity was 99%, 99%, 98%, 98% and 89%, respectively, conforming that the RT-PCR production in this study is authentically the sheep *TNNC2* cDNA.

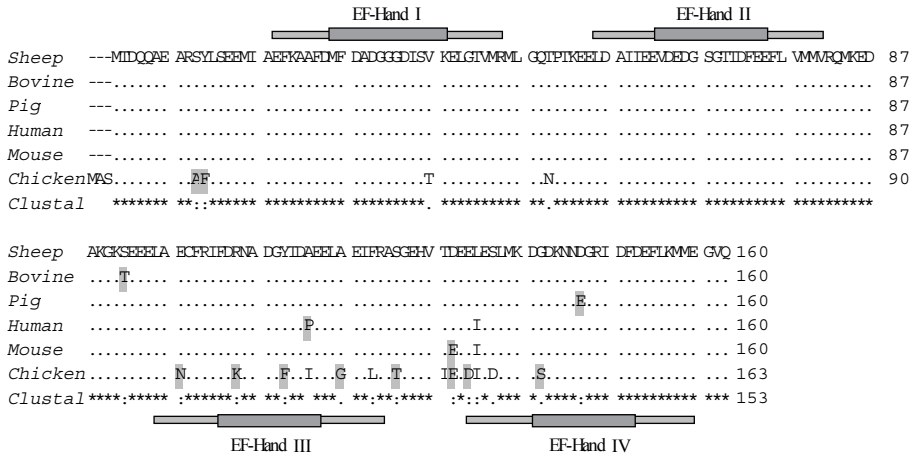

Fig. 2: Multiple alignment of amino acid sequence of TNNC2 in some species. The sequence of sheep was aligned with bovine, pig, human, mouse and chicken. Amino acids are shown in single letter code. The four EF-hand Ca^{2+} binding domains (I–IV, with the helixes surrounding the loop regions) are marked with boxes. "*" represented that the residues in that site are identical in all sequences.
(Vergleich der Aminosäuren-Sequenz von *TNNC2* bei einigen Spezies)

Polymorphisms of TNNC2 gene

Fragments of 399bp (Figure 3a) and 323bp were obtained from sheep genomic DNA by using of two pairs of primers TN2 and TN3. No polymorphism was observed in TN3 amplified products. However, three haplotypes were detected in TN2 amplicon which represented DD (−/−), DT (T/−) and TT (T/T) genotypes (Figure 3b). Sequenced results revealed that one SNP of *TNNC2* gene existed at the 7th bp (GTTA>GT-A) in intron 1 (Figure 3c). The distributions of genotypes and alleles in the three sheep populations were summarized in Table 2. The frequencies of T allele in the three sheep populations were calculated as 0.21 (Small-Tailed Han sheep), 0.23 (Tan sheep) and 0.29 (Inner Mongolia sheep). The genotype distributions of the three sheep populations were in agreement with Hardy-Weinberg equilibrium. There was a moderate polymorphism (0.25<P<0.5) (Table 2) in the three sheep breeds according to the standard of BOTSTEIN et al. (1980).

Table 2
Genetic diversity of *TNNC2* loci in three Chinese sheep populations
(Genetische Diversität des *TNNC2* bei drei Schafpopulationen aus China)

Index	SH	TS	IMS
Individuals	60	58	60
Individuals of $_{DD}$ genotype (Frequency)	38 (0.63)	34 (0.59)	31 (0.52)
Individuals of $_{TD}$ genotype (Frequency)	19 (0.32)	21 (0.36)	23 (0.38)
Individuals of $_{TT}$ genotype (Frequency)	3 (0.05)	3 (0.05)	6 (0.10)
Frequency of allele *D*	0.79	0.77	0.71
Frequency of allele *T*	0.21	0.23	0.29
PIC (polymorphism information content)	0.34	0.35	0.37
P value (Locus equilibrium χ^2 test)	0.705	0.969	0.530

SH=Small-Tailed Han sheep; TS=Tan sheep; IMS=Inner Mongolia sheep

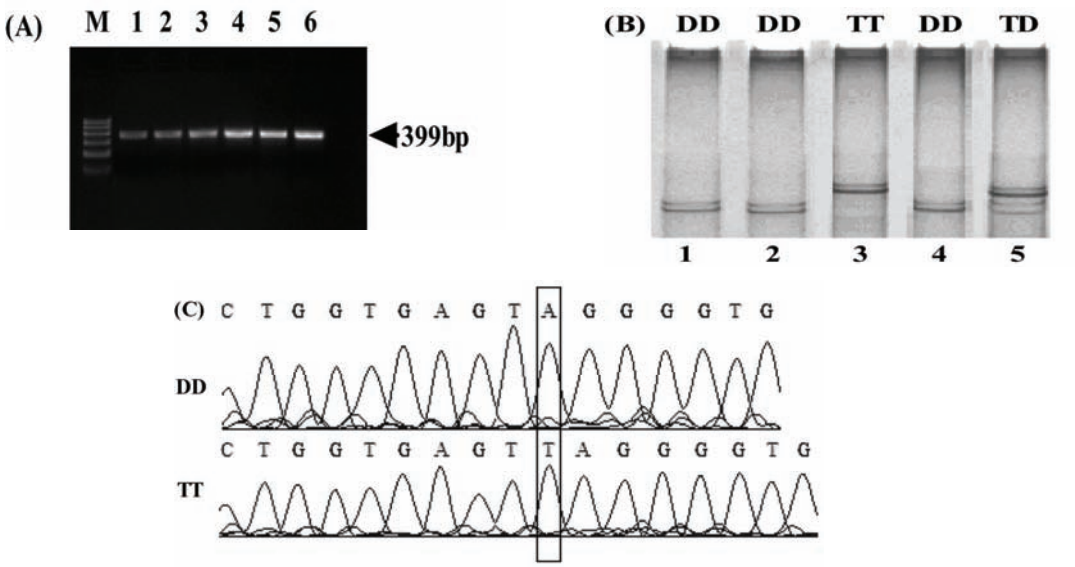

Fig. 3: Panel A shows the examination of amplicon of TN2 primers. M is the marker I (bands are 100, 200, 300, 400, 500 and 600bp). Panel B is the SSCP analysis of TN2 products; three haplotypes are detected based on their electrophoretic mobility on the gel. In lane 1, lane 2 and lane 4 the haplotypes are similar, named as DD. Lane 3 and lane 5 named as TT and TD genotype, respectively. Panel C is the sequence comparison of DD and TT genotypes. Block box site indicated the deletion of the T base pair in DD genotype.
(A: Gelbild des PCR-Fragments erzeugt mit Primern TN2,; B: SSCP-Gel; C: Sequenzabschnitt mit InDel)

Effects of TNNC2 Genotypes on meat quality traits

The associations of *TNNC2* genotypes with meat quality traits were shown in Table 3. Genotypes had no effects on 90 days-body weight, loin-eye area and meat color. The 90 days-hot carcass weight of DD and DT group were weightier than that of TT group ($P<0.05$). The difference between the DD and DT genotypes was not significant. Genotypes affected the shear force of sheep LT meat. Compared with DD and DT genotypes, TT had lower shear force ($P<0.05$), while no difference was found between DD and DT genotypes. It suggested that TT genotype conferred more tenderness of meat quality.

The LT muscle marbling score ranged from 1.78 to 2.61 (Table 3), sheep with TT genotype had significantly ($P<0.01$) higher marbling score than DD and DT genotypes, no difference were observed between DD and DT genotypes. The same pattern of differences of meat pH value was also found between the three genotypes (Table 3).

Table 3
The associations of *TNNC2* genotypes with LT meat quality traits in the sheep populations traits
(Assoziation von *TNNC2* mit Merkmalen der Fleischqualität im *M. longissimus tharocis*)

Traits	Genotypes of *TNNC2* (μ±SE)			F-Test	a	d
	DD (n=103)	DT (n=63)	TT (n=12)			
BWT_{90} (kg)	11.50±1.39	10.87±3.06	9.64±0.63	NS	−0.93	0.31
$HCWT_{90}$ (kg)	4.99^{a}±0.80	4.66^{a}±1.19	4.06^{b}±0.57	*	−0.47	0.13
LA (cm^2)	11.50±3.05	10.73±2.90	10.16±0.80	NS	−0.67	−0.09
SF (N)	26.16^{a}±2.90	26.48^{a}±5.37	22.61^{b}±2.47	*	−1.78	2.09
DL (%)	9.93^{A}±1.90	9.28^{A}±1.01	6.71^{B}±2.03	**	−1.61	0.96
MB	2.06^{A}±0.59	1.78^{A}±0.59	2.61^{B}±0.49	**	0.28	−0.56
L^*	40.56±4.64	42.50±3.54	42.78±2.64	NS	1.11	0.83
pH_{45}	6.00^{A}±0.49	5.52^{A}±0.59	6.75^{B}±0.39	***	0.38	−0.86

BWT_{90}= 90 days-body weight; $HCWT_{90}$ = 90 days-carcass weight; LA=Loin-eye area; SF= Shear force; DL= Drip loss rate; MB= Muscle marbling score; L^* = Meat color score; pH_{45} = pH value; a, d = additive effects and dominance effects, respectively, a = 0.5(TT-DD), d = DT-0.5(TT+DD); NS = non significance; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; LS-Means with unequal superscripts in a row are significant difference

The TT genotype had lower (P<0.01) drip loss rate compared with DD and DT genotypes, no significant difference was found between DD and DT genotypes.
Correlation coefficients (r) between sheep meat quality traits were presented in Table 4. 90 days-body weight of sheep demonstrated positive correlation with 90 days-carcass weight (r=0.64, P<0.01) and drip loss rate (r=0.38, P<0.05). Meanwhile, 90 days-carcass weight was positively correlated with shear force (r=0.41, P<0.05), but negatively with pH value (r=−0.43, P<0.05). Drip loss rate was positive correlation with pH value (r=0.39, P<0.05).

Discussion

The Small-Tailed Han sheep, Tan sheep and Inner Mongolia sheep are major meat sheep breeds in northern China. To take advantage the meat quality traits of the three breeds in selective breeding schemes, the characteristics and molecular markers of meat quality traits should be known, in which tenderness is the most important traits. Our results showed (Table 4) that the 90 days-carcass weight was positively correlated with shear force (r=0.41, P<0.05) in three Chinese sheep breeds, negatively correlated with meat tenderness subsequently. In ruminants, meat tenderness is thought to be influenced by the pattern of the animal's growth, in which a decrease in growth often exhibits much lower in carcass weight and more tenderness than growing faster (HARPER, 1999). It also suggested that selection for increasing sheep meat production decreased the meat tenderness, which agreed with the previous results of SHACKELFORD et al. (2003) and MARTÍNEZ-CEREZ et al. (2005).

Table 4
Pearson's correlation coefficients between meat quality traits of sheep
(Korrelationen zwischen Merkmalen der Schlachtkörper- und Fleischqualität)

	BWT_{90}	$HCWT_{90}$	LA	SF	DL	MB	L^*	pH_{45}
90 days-body weight (BWT_{90})	1	0.64**	0.05	0.22	0.38*	0.08	0.06	−0.25
90 days-carcass weight ($HCWT_{90}$)		1	0.12	0.41*	0.33	0.06	0.28	−0.43*
Loin-eye area (LA)			1	−0.08	−0.01	−0.14	0.20	−0.22
Shear force (SF)				1	0.23	−0.11	−0.08	−0.23
Drip loss rate (DL)					1	−0.09	−0.07	0.39*
Muscle marbling score (MB)						1	0.05	0.34
Meat color score (L^*)							1	−0.09
pH value (pH_{45})								1

*P<0.05, **P<0.01

The *TNNC2* was considered as a meat quality candidate gene in our study. The protein sequence analysis indicated that the *TNNC2* gene was conserved among different species (Figure 2), which related with its function. As an EF-hand protein (ELIZABETH et al., 2006), *TNNC2* is the key element in skeletal muscle contraction. EF-hand I and II located in the N-terminus of the protein are regulatory sites, and EF-hand III and IV located in the C-terminus are structural sites (Figure 2) (PUTKEY et al., 1991). Ca^{2+} binds to sites I and II, and *TNNC2* structure change initiates cascade interactions with thin filaments, resulting in an "on" state of *TNNC2*. The activated *TNNC2* then trigs strong myosin cross-bridge binding to actin, leading to muscle contraction (FARAH and REINACH et al., 1995).
Most SNP in intron or a silence mutation do not have a direct impact on production traits. The mutation located in intron 1 of *TNNC2* was firstly detected and affected

meat quality traits in sheep population in this study. This association could be due to the reasons as follows:

1. Others mutation occurred within 3′ or 5′ flanking regulatory regions.
2. Another genes were involved in the regulation of TNNC2 expression.

There were some evidences that subunits of the troponin complex, TnC and troponin I (TnI), were involved in defining the pH sensitivities of striated muscle, and played a central role in conferring pH sensitivity of Ca^{2+} activated contraction in muscle (METZGER, 1996). The acidic pH decreased Ca^{2+} binding to TnC, and the magnitude of the pH effect could increase when TnC complexed with TnI (SOLARO et al., 1989); in addition, changes in pH significantly altered the release kinetics of TnC-TnI interaction (VALERIA et al., 2006). Additionally, the skeletal fast TnI is expression during porcine myogenesis (MRUÁNI et al., 2007). The SNP indirectly affected meat quality traits by being linked with other genes or quantitative trait locus (QTL) that directly influenced the meat quality traits. However, in livestock, only porcine *TNNC2* gene has been fine mapped on chromosome 17 located at 17q2.1-2.2 by in situ hybridization (ZAMBONELLI et al., 2000). Interestingly, chromosome 17 of porcine had many QTL and six QTL for meat quality been mapped (MALEK et al., 2001), in which contained 63 genetic markers for meat quality traits (RAMOS et al., 2006). It is possible that *TNNC2* gene linked with those markers for meat quality, though the meat quality effects of porcine *TNNC2* has not been reported. Furthermore, compared with other livestock animals, there were limited literatures in QTL identification of sheep. The mapping and the linkage characterization of sheep *TNNC2* gene need be studied in future. Moreover, the exact mechanism of *TNNC2* gene polymorphism contributing to meat quality remains further investigation.

In conclusion, we cloned the full coding region of sheep *TNNC2* gene, analyzed the associations between SNP of sheep *TNNC2* and meat quality traits in 178 lambs. Significant association of different genotypes with meat shear force ($P<0.05$), meat marbling score ($P<0.01$) and implied that the T allele of *TNNC2* had a positive effect on sheep meat tenderness. We suggest that genotype TT could be regarded as molecular marker for tenderness and marbling score.

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