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Full Length Research Paper

Cloning, structural analysis and expression of cardiac troponin C (*TNNC1*) gene in goat

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Cardiac troponin (TNNC1) is the calcium-binding subunit of the myofibril thin filament that regulates excitation-contraction coupling in cardiac muscle. In the study, we cloned *TNNC1* gene of goat for the first time (GenBank accession number: HQ640744) and analyzed its tissue expression. Results indicate that TNNC1 was a 161-amino acid polypeptide that had been highly conserved during evolution. Its nucleotide sequence was high and was similar in various animals ranging from 97.94 (cow) to 83.33% (African clawed frog), and the identity of the encoded amino acids varied from 100 (cow) to 92.55% (smelt). Fluorescence quantitative PCR analyses revealed that the *TNNC1* gene was selectively expressed in the muscular tissues of goat, was expressed in cardiac tissue and slow skeletal muscle (soleus), but was not expressed in fast skeletal muscle (longissimus muscle, gluteus maximus) and brain, kidney, lung or liver of goat. The amino acid residue of Ca^{2+} binding site II is identical in mammals, aves and fish. This study may provide more insight into the molecular structure, expression patterns and evolution of *TNNC1* gene in animal.

Key words: TroponinC1 gene, molecular cloning, structural analysis, expression, goat.

INTRODUCTION

Muscle fibre can be classified according to their contractile nature (Fonseca et al., 2003). Thus, the genes involved in myoblast differentiation and muscle contraction are considered usually as potential candidate genes for meat quality (Te Pas and Soumillion, 2001). Troponin (TNNC) is the calcium-binding subunit of the myofibrillar thin filament that regulates excitation-contraction coupling in heart and skeletal muscle (Kretsinger, 1980). There are two isoforms of TNNC found in vertebrate; *TNNC2* express in fast skeletal muscle and *TNNC1* express in slow skeletal muscle and cardiac muscle. The difference of the two isoforms is that TNNC2 is activated by Ca^{2+} binding site I and II on the N-terminal, while TNNC1 is activated by Ca^{2+} binding site II (O'Connell et al., 2006).

Cardiac troponin (TNNC1), which has three functioning Ca^{2+} binding sites, is a prominent member of the troponin complex. Each of Ca^{2+} binding sites is characterized by a 12-residue Ca^{2+} binding loop that is interposed between a pair of α-helices. Each of the Ca^{2+} binding loops is rich in acidic amino acids (Asp and Glu) that are responsible for the coordination of a single Ca^{2+} (Johnson et al., 1980). TNNC1 is a 161-amino acid protein that is 67% homologous to TNNC2 at the amino acid level, diverging predominantly at the N-terminal domain of the protein (Schreier et al., 1990; Babu et al., 1992). The integral structure of TNNC1 consists of a N-terminal and a C-terminal globular domains connected by a flexible central helix, giving it a dumbbell-like shape, and each globular domain occupies a pair of EF-hand motifs which can bind to metal ions (Babu et al., 1992).

The diameter and type of muscle fiber will directly influence on meat tenderness, in which the types of myofiber can be classified according to their contractile nature (Fonseca et al., 2003). As a subunit of troponin complex, *TNNC* gene was expressed during the myoblast differentiation and skeletal muscle development (Bucher et al., 1988). Cardiac troponin (TNNC1) plays a critical role in soleus contraction and systolic (Farah and Reinach, 1995). To understand the molecular structure, expression patterns and evolution of *TNNC1* gene in animal and applied to goat production, the *TNNC1* was considered as a candidate gene with meat quality in our study.

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Table 1. Primers used to amplify the TNNC1 gene of Tianfu goat.

Parameter	Primer	Sequence (5'-3')	Amplicon size	NCBI accession number	Application
P2	F	GATGGCAGCGGCACAGT	109	HQ640744	Expression
	R	TGCGGAAGAGGTCAGAAAG			
β-actin	F	GTCACCAACTGGGACGACA	208	U39357	RT-PCR and control
	R	AGGCGTACAGGGACAGCA			
P1	F	CCTGTGAGTCGCCAGTATG	522	HQ640744	Amplification of cDNA and clone

MATERIALS AND METHODS

Experimental animals

20 ewes of Tianfu goats, a new meat goat breed in China, were slaughtered at six month, one year, two years and adult (five for each group). The tissue samples of the carcasses including heart, liver, lung, kidney, brain, longissimus muscle, gluteus maximus and soleus were collected immediately after slaughter and frozen in liquid nitrogen jar for total RNA extraction.

Cloning of TNNC1 gene

Total RNA was extracted from tissues sample using Trizol (TaKaRa, Dalian, Chian). According to the procedure of the synthesis kit, the first strand cDNA was synthesised from total RNA. According to Bos taurus *TNNC1* gene sequence (GenBank Accession No. BC102995.1), a pair of primer was designed with software primer 5.0 in the conserved region. The primers are shown in Table 1 (P1). Then using the primers, a cDNA fragment was amplified by PCR with first strand cDNA as templates. The PCR started with pre-denaturation at 95℃ for 5 min, followed by 35 cycles (94℃, 40 s; 55℃, 40 s; 72℃, 50 s), and ended with final extension at 72℃ for 10 min. Then, the PCR products were separated by electrophoresis on a 2% agarose gel, and purified by the PCR Rapid Purification Kit (BioDev-Tech). Purified PCR products were cloned by pMD18-T vector (TaKaRa, Dalian) and were sent to TaKaRa Biotechnology (Dalian) Co. Ltd. for sequencing.

Sequence analysis

Using BLAST software, we performed a sequence similarity analysis for the *TNNC1* nucleotide and amino acid sequences. Also, we determined a theoretical molecular weight and isoelectric point using peptide mass software. The phosphorylation site was estimated by NetPhos 2.0 Server. Multiple alignment analysis was conducted by the program CLUSTAL X and Bio-Edit software, and the phylogenetic analysis was performed by MEGA version 4.0 with neighbor-joining method. The SWISS-MODEL was used to model the protein conformation, which was viewed in the Swiss Pdb Viewer (Schwede et al., 2003).

Tissue expression analysis

Tissue expression pattern of *TNNC1* was performed by fluorescence quantitative PCR (RT-PCR) using goat *β-actin* gene as an internal expression control. The primers of *β-actin* (Table 1), which were used to adjust the concentration of cDNA, were designed based on the nucleotide sequence deposited in the Gen Bank (Accession No. U39357). The quantitative primers of *TNNC1* are shown in Table 1. Total RNA was extracted from the heart, liver, lung, kidney, brain, longissimus muscle, gluteus maximus and soleus, respectively. The amplifications were performed in a 20 µl reaction volume containing 12.5 µl of 2× SYBR Premix ExTaq (TaKaRa, Dalian), 0.5 µl of each primer, 2 µl of diluted cDNA, and 4.5 µl ddH_2O. The PCR amplification was carried out as follows: 95℃ for 10 s followed by 40 cycles of 95℃ for 5 s and 60℃ for 34 s. The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression level of the gene in goat (Livak and Schmittgen, 2001).

RESULTS

cDNA cloning and sequence analysis of TNNC1

A 522 bp sequence of *TNNC1* gene was obtained by homologous cloning using the cDNA from the cardiac muscle of Tianfu goat (GenBank accession No. HQ640744). The sequence analysis showed that CDS of *TNNC1* was 486 bp with 161 encode amino acids residues (Figure 1), 18.42 kDa predicted molecular weight and 4.05 isoelectric points. The total numbers of amino acid were 63 including 46 negative charge residues and 17 positive charge residues, and that indicated the protein took negative electricity. There were seven phosphoric sites successfully predicted by the neural network (Figure 1). There was no signal peptide in the protein. Hydrophobic correlation analysis showed that the minimum was -2.42 and the maximum was 1.25 (Figure 2). The result indicate that the protein hydrophobicity ability was strong. The secondary structure of the protein was mainly with α-helix, random coil and β-sheet, and the β-sheet only exists in the area of EF-Hand Site I.

Characteristics of deduced protein and phylogenetic analysis of TNNC1

Comparison of the TNNC1 from goat with nine other mammals, aves, amphibian and fish revealed that the TNNC1 had a high identity with the reported animals (Figures 3 and 4). The identity of nucleotide of this TNNC1 with these animals varied from 97.94 (cow) to 83.33% (African clawed frog). The amino acid sequence

Figure 1. The nucleotide and deduced amino acid sequences of TNNC1 from goat. The emerald green indicate binding site of P1, the yellow indicate binding site of P2 and the red indicate phosphoric sites (Ser, Thr and Tyr).

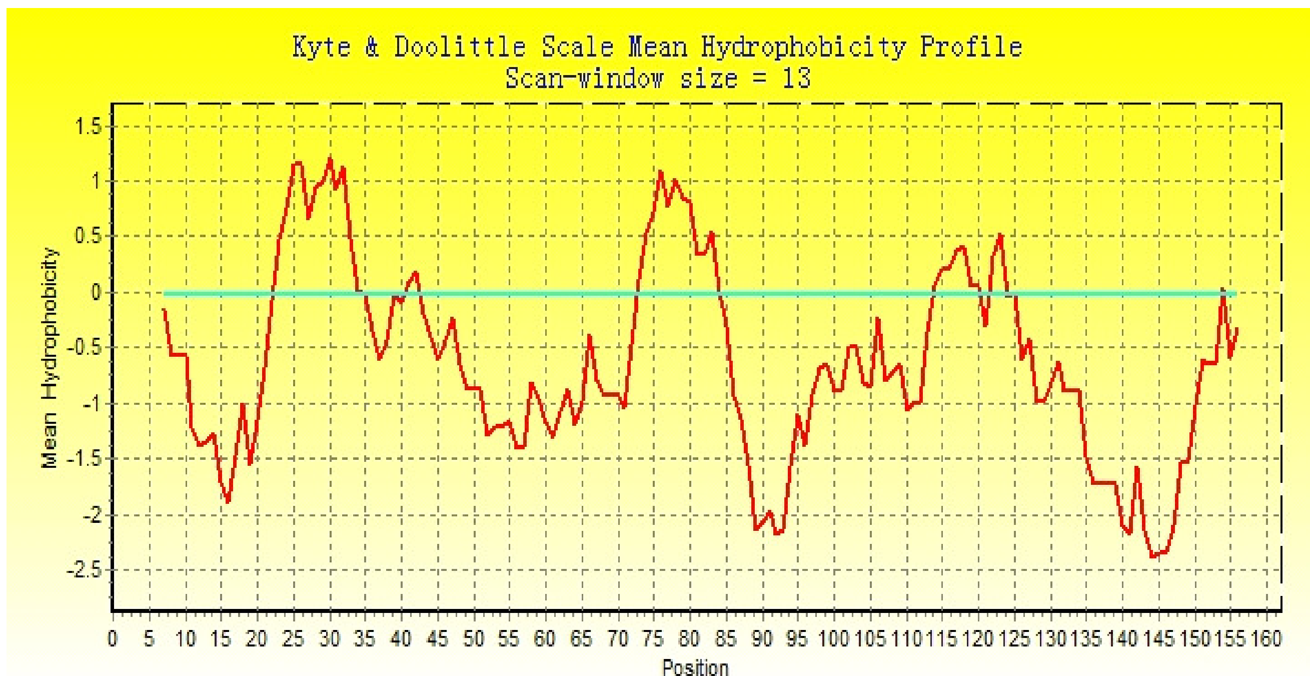


Figure 2. The hydrophobicity profile of TNNC1 from goat. The horizontal scale indicate the number of amino acid residues and the vertical one was the relative hydrophobic scale. Points below the zero horizontal line correspond to hydrophobic region, and points above the line were hydrophilic region.

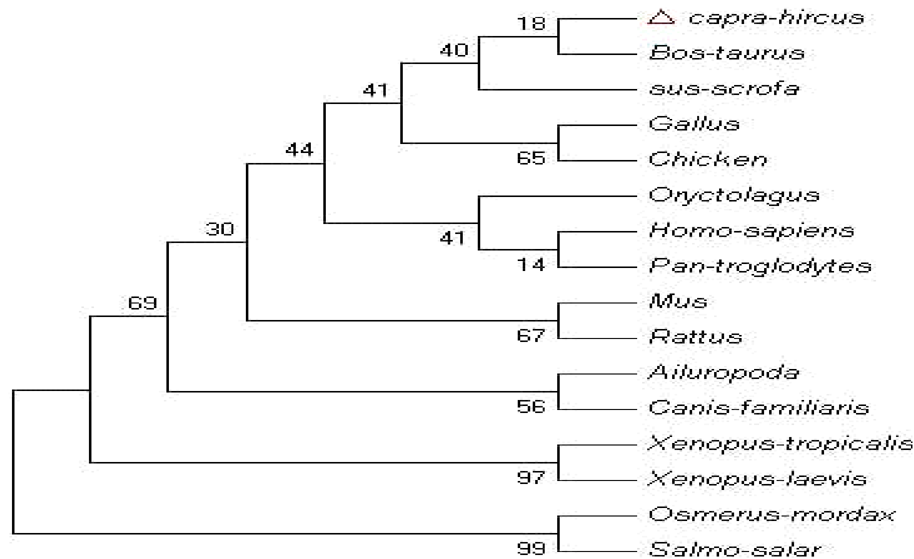


Figure 3. Phylogenic tree of TNNC1 of animal. The GenBank accession numbers of the sequences are listed in the Table 2.

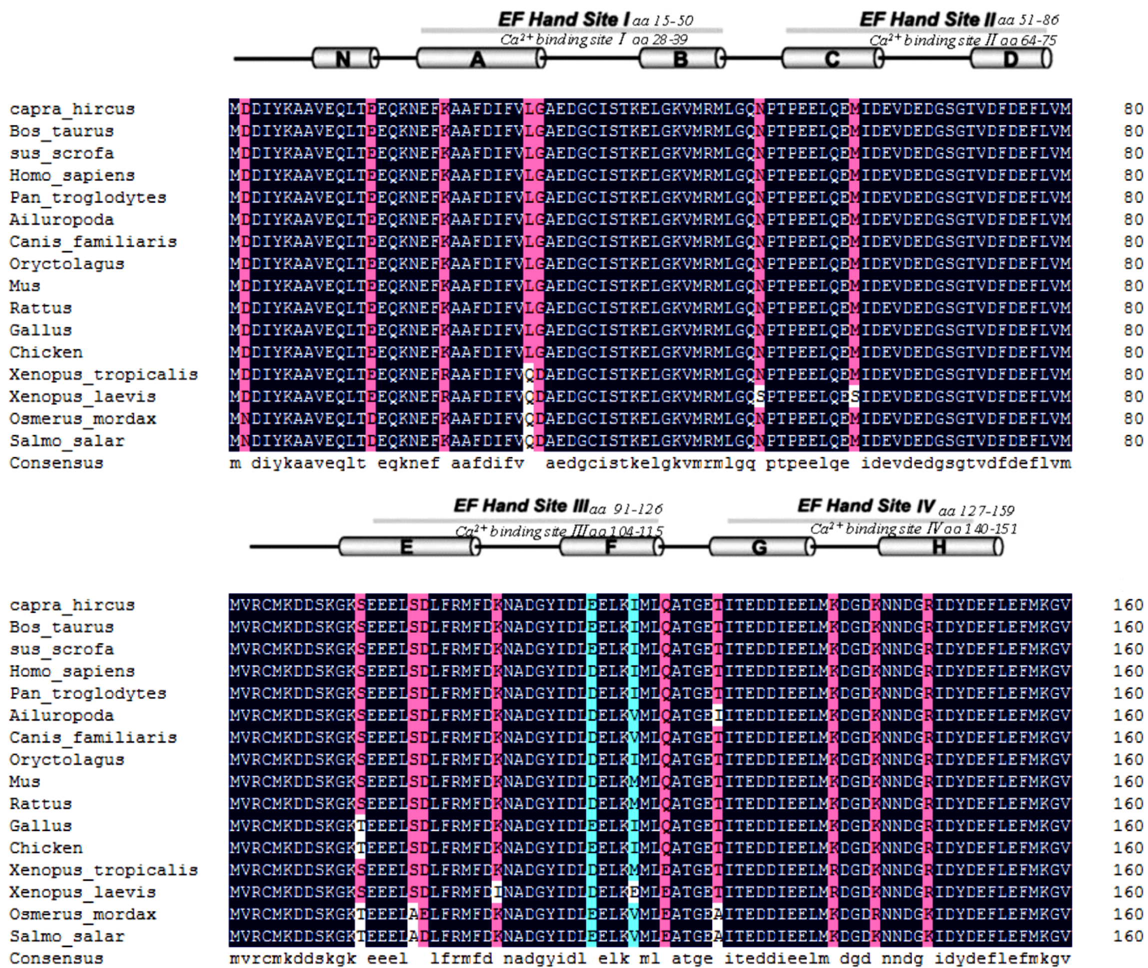


Figure 4. The sequence comparison of TNNC1 in representative species. The Ca^{2+}-coordinating positions in each EF-hand site are shown above the sequences, and the helices are labeled. The two interesting amino acid residue are highlighted by blue.

Table 2. List of TNNC1 sequences used in the analyses.

Organism	Common name	Ribonucleotide identity (%)	AA Identity (%)	Taxonomy	Identifier	Database
Capra hircus	Goat			EM	HQ640744	Genbank
Bos taurus	Cow	97.94	100.00	EM	NM001034351.1	Genbank
Sus scrofa	Pig	94.03	100.00	EM	NM001130243.1	Genbank
Homo sapiens	Human	94.03	99.38	EM	AK313743.1	Genbank
Pan Troglodytes	Gorilla	94.03	99.38	EM	XM001172150.1	Genbank
Ailuropoda Melanoleuca	Panda	93.21	98.14	EM	EF410079.1	Genbank
Canis familiaris	Dog	93.00	98.76	EM	XM533799.2	Genbank
Oryctolagus cuniculus	Rabbit	90.74	99.38	EM	XM002713240.1	Genbank
Mus musculus	Mouse	89.71	98.76	EM	XM009393.2	Genbank
Rattus norvegicus	Rat	89.71	98.76	EM	NM001034105.1	Genbank
Gallus gallus	Chicken	88.07	99.38	bird	NM205133.1	Genbank
Chicken	Chicken	88.07	99.38	bird	M16024.1	Genbank
Osmerus mordax	Smelt	87.65	92.55	fish	BT075376	Genbank
Salmo salar	Atlantic salmon	87.04	93.79	fish	BT047422.1	Genbank
Xenopus tropicalis	Western clawed frog	84.16	95.65	AM	NM001004776.1	Genbank
Xenopus laevis	African clawed frog	83.33	93.79	AM	NM001086254	Genbank

of the TNNC1 of goat had a 100% identity with cow and pig (Table 2). There were three amino acids missense mutation in the 10 mammals. Glu^{115} in artiodactyla (cow, goat and pig) and val^{119} in carnivora (panda and dog) both belong to conservative substitution. However, Ile^{127} in giant panda is non-conservative properties substitution.

The 3D structural model of TNNC1

SWISS-MODEL server, a fully automatic procedure, was used to construct a 3D structural model of caprine TNNC1 using bovine TNNC1 as the template (PDB accession, 1a2xA) (Figure 5A) and the Ca^{2+} binding sites were marked out by the SPDBV 4.01 molecular-graphics program (Figure 5B).

Relative expression pattern of TNNC1 in goat

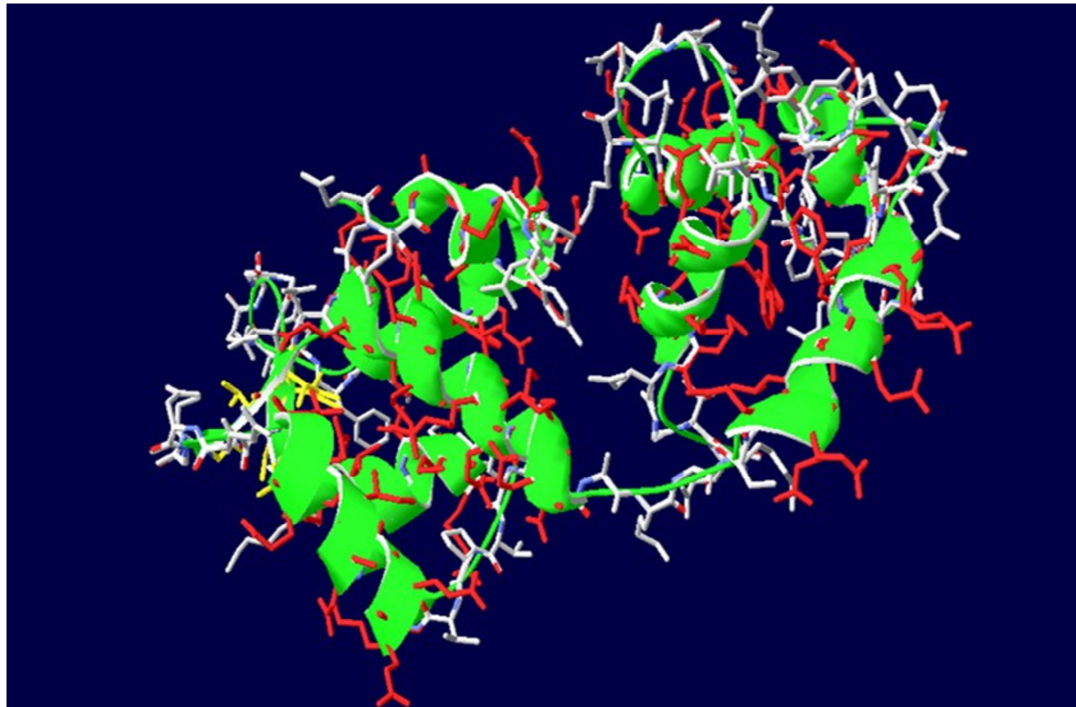
We performed fluorescence quantitative PCR to analyze its mRNA transcription levels. Results indicate there were some differences in *TNNC1* transcription levels between the four developmental stages of the goat (Figure 6A). The transcription level of *TNNC1* was highest in six-month old goats. The expression amount of *TNNC1* was highest in heart and soleus. There were no expression of *TNNC1* in the longissimus muscle and other non-muscle tissue (Figure 6B).

DISCUSSION

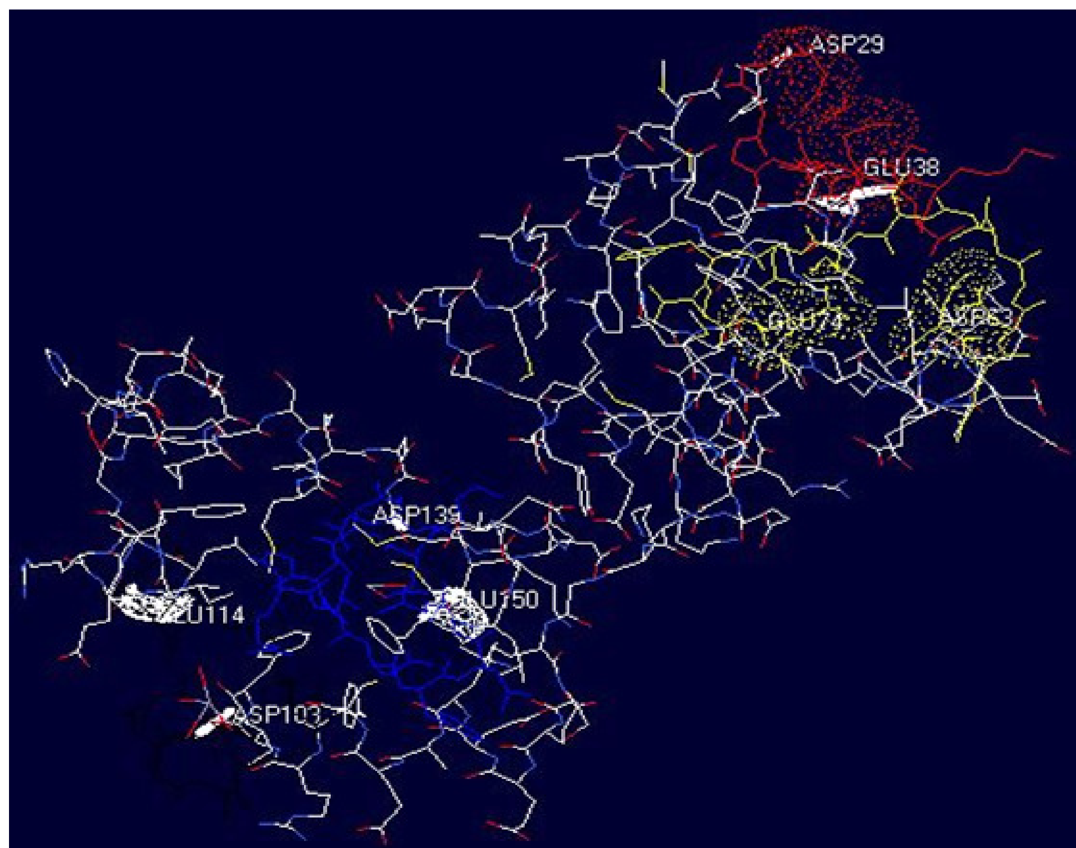
The genes involved in skeletal muscle development, myoblast differentiation or skeletal muscle contraction are considered usually as potential candidate genes for meat quality (Te Pas and Soumillion, 2001). Meat quality is associated with fibre types of muscle (O'Halloran et al., 1997), in which the fibre types can be classified according to their contractile nature (Fonseca et al., 2003). Thus, cardiac troponin (TNNC1) plays a critical role in physiologic functions of muscle contraction (Farah and Reinach, 1995). The study reveals that the encoded amino acids of caprine TNNC1 was highly homologous with that of other species; the identity of TNNC1 among goat, cow and pig was 100%. It suggest that *TNNC1* gene was conserved during evolution.

TNNC1 consists of two globular domains connected by α-helixes linker. Both N-terminal and C-terminal domains contain two EF-hand Ca^{2+} binding sites, and each EF-hands consist of two α-helixes connected by a flexible loop. The helices and loop in the motif each contain 12 amino acid residues (Nakayama et al., 1992). In our study, this motif was also presented in the deduced amino acid sequence of the TNNC1 in goat. We found that the TNNC1 of goat included four Ca^{2+} binding sites, and contained only three functional binding sites. The four calcium binding sites of goat began with Asp and ended at Glu (Figure 5B). Comparison of calcium-binding site residues in the amino acid sequences of various species indicates that TNNC1 of goat was a member of the EF-hand superfamily.

Binding of Ca^{2+} to the low-affinity sites of TNNC, particularly Ca^{2+} binding site II, is the trigger event for muscle contraction (Gillis et al., 2007; Leszyk et al., 1988). We found there were only conservative replacement of the amino acids of the functional binding domains of TNNC1 in mammals, aves and amphibians. It



(A)



(B)

Figure 5. The 3D-structural model of caprine TNNC1. A, the structure of alpha-helix, beta-strand and random-coils; B, the structure of backbone. The Ca^{2+} binding sites I, II, III and IV are highlighted by red, yellow, black and blue, respectively.

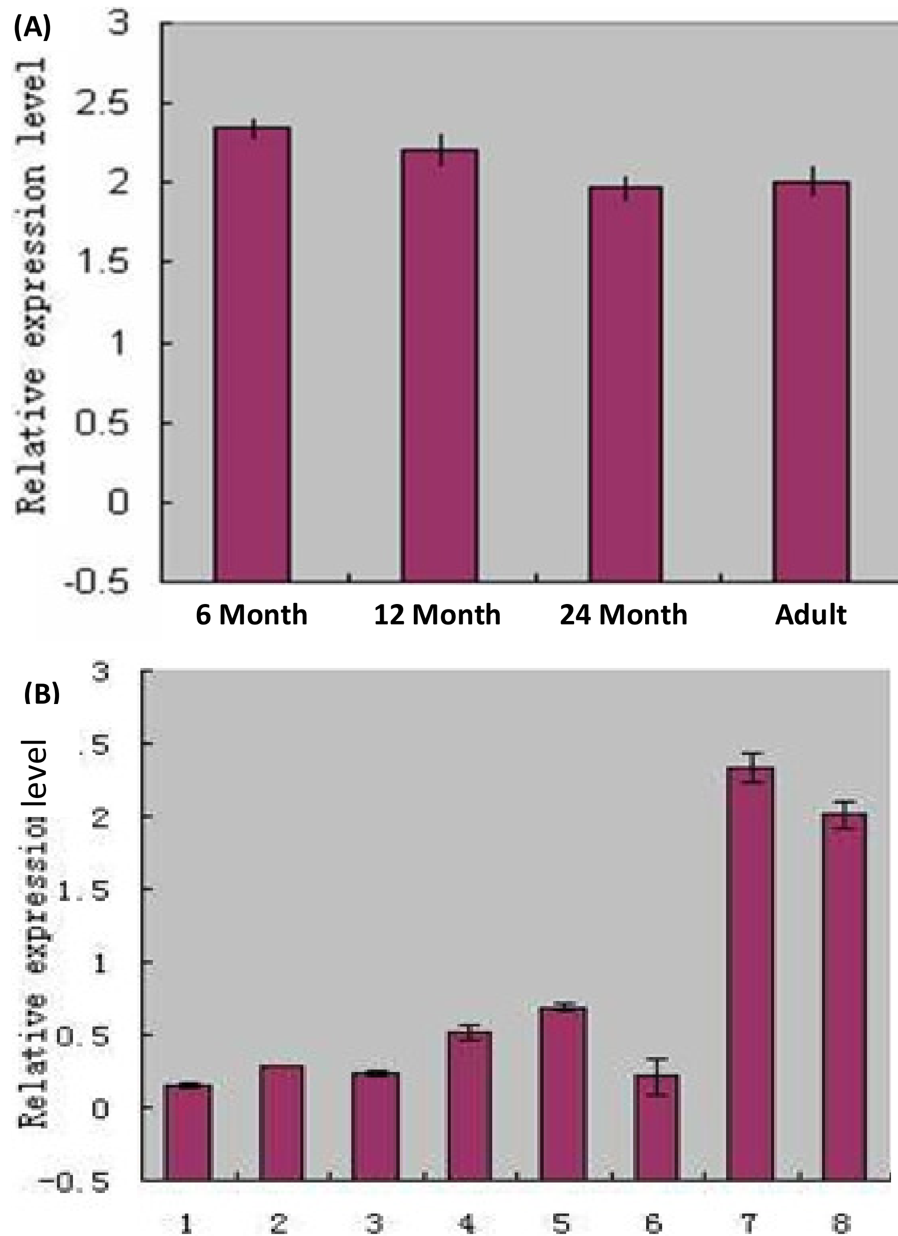


Figure 6. Histogram showing the expression of *TNNC1* gene in goat. The vertica1 axis indicate the value of the *TNNC1* mRNA. (A) The horizontal axis indicate the various growth stages of *TNNC1* in cardiac muscle; (B) The horizonta1 axis indicate the various tissues of yearling. Bars 1 to 8 represents liver, brain, kidney, gluteus maximus, lung, longissimus muscle, heart and soleus,, respectively.

suggests that there were rigid structure-functional requirements for cardiac troponin C to operate excitation-contraction coupling of muscle.

Studies by Dhoot et al. (1979) and Parmacek et al. (1990) showed that *TNNC1* was only expressed in cardiac and slow skeletal muscle, whereas, *TNNC2* was found exclusively in fast skeletal muscle. Other studies also demonstrate that *in vivo*, the *TNNC1* gene is expressed only in slow skeletal (soleus) and cardiac muscle and is not expressed in brain, lung, liver, kidney, or testis (Parmacek and Leiden, 1989).

In this study, the *TNNC1* gene of goat was expressed at a high level in cardiac muscle and soleus, and the highest expression was in cardiac muscle of goat. Moreover, further results show that there were high expression in cardiac muscle in various growth stage including six, 12, 24 months and adult. Correspondingly, the gene was expressed at a low level in liver, brain,

kidney, gluteus maximus, lung and longissimus muscle of goat. It is conducive to understand the molecular mechanisms of the gene for regulating fast skeletal muscle and cardiac muscle. Furthermore, the study may provide more insight into the molecular structure, expression patterns and evolution of *TNNC1* gene in animal, and provide an important theoretical basis for further research on the application of the gene in goat production.

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