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Molecular Characterization and Expression Analysis of *Myf*6 Gene in Yak (*Bos grunniens*)

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Abstract

To reveal the sequence characteristic and expression pattern of *Myf*6 gene in Jiulong yaks (*Bos grunniens*), a full-length cDNA of *Myf*6 was cloned from yak muscle tisssue by RT-PCR. The cDNA obtained was 774 bp nucleotide (nt) long with an ORF of 729 bp which encoding 242 amino acids. Compared with Myf6 protein sequences of *Bos Taurus*, *Capra hircus*, *Sus scrofa*, *Homo sapiens*, *Equus caballus*, *Oryctolagus cuniculus*, and *Mus musculus*, the homology of amino acid sequences were higher (100 %-94 %), but lower in Zebrafish (60 %). Semi Quantitative (SQ) RT-PCR analysis showed that *Myf*6 gene expression was observed mainly in *longissimus* muscle, and trace expression in spleen, but not be detected in heart, liver, kidney, and adipose tissues. The expression of *Myf*6 gene in *Longissimus dorsi* of male yaks increased with age. The levels of *Myf*6 mRNA in *Longissimus* muscles of 3.5-5.5 yr and over 9.0 yr yaks were significantly higher than that of 0.5 yr yaks ($p<0.05$). These results suggest that *Myf*6 may play an important role in maintaining skeletal muscle phenotype of yak.

Keywords

Yak, *Myf*6 cDNA, Molecular Characterization, Expression Analysis, *Longissimus dorsi*

1. Introduction

The yak (*Bos grunniens*) is an iconic symbol of Tibet and of high altitude. More than 14 million domestic yaks provide the basic resources (such as meat, milk, transportation, dung for fuel and hides for tented accommodation) that are necessary for Tibetans and other nomadic pastoralists in high-altitude environments [1]. According to Wiener et al. (2003), the total yak population is estimated to number around 14.2 million, of which 13.3 million are in Chinese territories and the number are said to be increasing in some areas of China.

Myogenesis is a multistep process in which multipotent precursor cells give rise to myoblasts that subsequently withdraw from the cell cycle, and differentiate and fuse into multinuclear myotubes and then myofibers[2,3]. The muscle regulatory factors (MRFs), consisting of MyoD, myogenin, Myf5 and Myf6 (MRF4), are considered to be the master regulators of skeletal myogenesis. Each gene of this family is composed of three exons and share homology within the region coding for bHLH domain. MyoD and Myf5 are required for the specification and proliferation of myoblasts [4-7]. MyoG and Myf6 mainly function during fusion of myoblasts into multinucleate myofibers in the animal [8-11]. As an important member of MRF family, Myf6 is involved in the processes of differentiation and maturation of myotubes during embryogenesis and continues on a relatively higher level than the other myogenic regulatory factors in adult muscle, affecting muscle fiber phenotype and maintaining the differentiated phenotype[12-14]. Myf6 tends to be expressed more highly in muscle tissue of the lean selection line which has higher lean mass and expression of Myf6 in the thicker muscle fibers for maintenance[14,15]. Therefore, Myf6 is regarded as the principal factor influencing skeletal muscle phenotype [16] and one promising candidate gene for growth- and meat quality-related traits in livestock [17]. Therefore in this study, we cloned the *Myf*6 gene sequence of Jiulong yak, and examined the expression profiles in various yak tissue samples of different growth stages in order to highlight the Myf6 roles in the molecular basis of meat quality and growth in yaks.

2. Materials and Methods

2.1. Sampling

Heart, liver, kidney, spleen, *Longissimus* muscles and adipose tissues were taken from healthy male yaks at ages of 0.5 yr (n=5), 3.5-5.5 yr (n=5) and over 9.0 yr (n=5). All of the samples were promptly frozen and stored at -80 ℃ until analysis. This experiment was conducted according to the guidelines of Chinese government for the use of experimental animals including animal welfare and conditions.

2.2. cDNA Cloning of Yak *Myf6* Gene

Total RNA was isolated from the muscle tissue of Jiulong yak using Trizol (Invitrogen) according to the manufacturer's recommendations. The quality of RNA samples were detected by ultraviolet spectrophotometer. First strand cDNA was synthesized using M-MLV reverse transcriptase (Thermo), and used as the template for PCR. The PCR primers were designed based on bovine *Myf6* sequence in GenBank (BC142159) as follows: F: 5'-AGAGAACATGATGATGGACCTT-3' and R: 5'-GATCTTCCTGCTCCGTGG-3'. The PCR conditions were as follows: 94 ℃, 3 min, then 39 cycles of 30 s at 94 ℃, 35 s at 56.6 ℃, 1.0 min at 72 ℃; 7 min at 72 ℃. The PCR product was purified and cloned into pMD 18-T Vector (TaKaRa Biotechnology (Dalian) Co., Ltd.). Three positive clones were sequenced from both strands.

2.3. Bioinformatics Analysis of *Myf6* Gene Sequence of Jiulong Yak

The open reading frame (ORF) of *Myf6* gene of Jiulong yak was identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The isoelectric point and molecular weight of the protein deduced from the nucleotide sequence were analyzed by ExPASy (http://www.expasy.org/tools). The conservative domain was predicted by NCBI tools (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and the signal peptide of the protein was predicted by SignalP 4.0 (http://www.cbs.dtu. dk/services/SignalP/) [18]. Amino acid sequence similarity analysis was performed by BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit 5.0.6[19], phylogenic tree was constructed by MEGA 6.0[20].

2.4. Tissue Expression of *Myf6* Gene of Jiulong Yak

Quantization of *Myf6* mRNA level in different tissues of male yaks at ages of 3.5-5.5 yr (n=5) were assessed by RT-PCR using *β-actin* as internal control. Total RNA and cDNA were prepared from heart, liver, kidney, spleen, *Longissimus* muscles and adipose tissues of Jiulong yaks as mentioned above. The primers were designed according to *Myf6* mRNA sequences of yak (BT030480). *Myf6*-F: 5'-TCCAGGGGGCTCGTGATAA-3', *Myf6*-R: 5'-ACGCAGGGGAGTTTGTGTTC -3'. The *β-actin* primers were designed based on the sequence (BT030480). *β-actin* -F: 5'- CCCATCTA TGAGGG GTACGC-3', *β-actin* -R: 5'-CCTTGATGTCACGGACGATTT -3'. Amplification was performed using the following cycling parameters: 94 ℃ for 2 min, 33 cycles of 94 ℃ for 30 s, 60 ℃ (for *Myf6*) /54 ℃ (for *β-actin*) for 30 s , 72 ℃ for 30 s and 72 ℃ for 1 min. After the reaction, PCR products were analyzed on 1.0% (w/v) agarose gels.

2.5. Time-Series Expression of *Myf6* Gene in *Longissimus* Muscles of Jiulong Yak

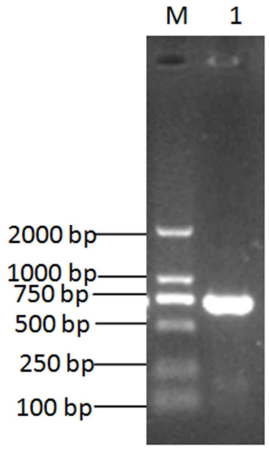
Fluorogenic quantitative PCR assay was developed for the quantization of *Myf6* mRNA level in *Longissimus* muscles of male yaks at ages of 0.5 yr (n=5), 3.5-5.5 yr (n=5), and over 9.0 yr (n=5). The primers for *Myf6* and *β-actin* were the same as above. The amplification mixture contained 10μl SYBR® Premix Ex Taq^{TM} (2×) (TaKaRa Biotechnology (Dalian) Co., Ltd.), 1μl of RT reaction mix, 0.5μL of 10 mmol/L each of primers and add ddH_2O to 20μL. The PCR conditions were as follows: one cycle of 1 min at 95 ℃; 45 cycles of 30 s at 95 ℃, 30 s at 60 ℃ (for *Myf6*) /54 ℃ (for *β-actin*), 30 s at 72 ℃. Each sample was run in duplicate.

2.6. Statistical Analysis

Data were analyzed using SPSS 17.0. Values were expressed as Mean ± SE. The developmental pattern difference of *Myf6* was assayed by one-way ANOVA, and significance level was set at $p < 0.05$. The threshold cycle was analyzed using the $2^{-\triangle\triangle Ct}$ method [21].

3. Results

The nucleotide sequence of cDNA of Jiulong yak *Myf6* was 774 bp (Fig.1) (GenBank accession No: KC184119) and contained an open reading frame of 729 nucleotides, encoding a predicted protein of 242 amino acids with classic bHLH domain and no signal peptide (Fig.2 and 3). *Myf6* cDNA of Jiulong yak is same to the *Bos taurus* Myf6 gene sequence in GenBank(NP_861527.1). The deduced molecular weight was 26.98 KD and the theoretical pi was 5.64.



M, DNA marker DL 2000; 1, PCR amplification product

Fig. 1. *RT-PCR amplification of Myf6 gene from muscle of Jiulong yak*

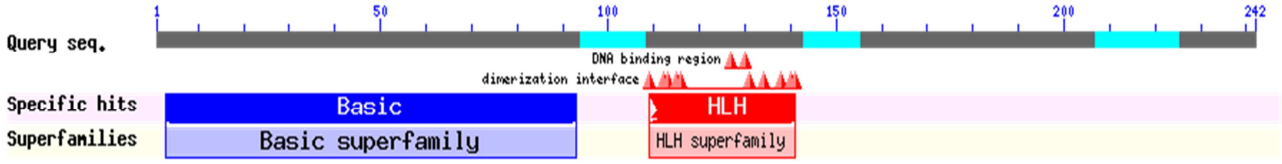


Fig. 2. *Prediction of biological function of the deduced amino acid sequence of Jiulong yak Myf6*

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1      AGAGAACATGATGATGGACCTTTTTGAAACTGGCTCCTATTTTTTCTACTTGGACGGGGA
              M  M  M  D  L  F  E  T  G  S  Y  F  F  Y  L  D  G  E

61     AAATGTTACCCTGCAGCCCTTAGAAGTGGCAGAGGGCTCTCCTCTGTATCCAGGGAGTGA
         N  V  T  L  Q  P  L  E  V  A  E  G  S  P  L  Y  P  G  S  D
121    TGGTACCCTGTC GCCCTGCCAGGACCAAATGCCCCCAGAAGCCGGGAGCGACAGCAGCGG
         G  T  L  S  P  C  Q  D  Q  M  P  P  E  A  G  S  D  S  S  G
181    AGAGGAACATGTCCTGGCGCCCCCAGGCCTGCAGCCTCCCCACTGTCCCGGCCAATGTCT
         E  E  H  V  L  A  P  P  G  L  Q  P  P  H  C  P  G  Q  C  L
241    GATCTGGGCTTGCAAGACCTGCAAGAGAAAATCTGCCCCCACCGACCGGCGGAAGGCCGC
         I  W  A  C  K  T  C  K  R  K  S  A  P  T  D  R  R  K  A  A
                        Basic domain
301    CACCCTGCGCGAGAGGCGGCGGCTCAAGAAAATCAACGAGGCCTTCGAGGCACTGAAGCG
         T  L  R  E  R  R  R  L  K  K  I  N  E  A  F  E  A  L  K  R
361    ACGGACTGTGGCCAACCCCAACCAGAGGCTGCCCAAGGTGGAGATTCTGCGGAGCGCCAT
         R  T  V  A  N  P  N  Q  R  L  P  K  V  E  I  L  R  S  A  I
         Helix-loop-helix domain

421    TAACTACATCGAGCGGTTGCAGGACCTGCTGCACCGGCTGGATCAGCAGGACAAAATGCA
         N  Y  I  E  R  L  Q  D  L  L  H  R  L  D  Q  Q  D  K  M  Q
481    GGAGTTAGGGGTGGACCCCTTCAGCTACAGACCCAAGCAAGAAAATCTTGAGGGTGCGGA
         E  L  G  V  D  P  F  S  Y  R  P  K  Q  E  N  L  E  G  A  D
541    TTTCCTGCGCACCTGCAGCTCCCAGTGGCCAAGTGTTTCGGATCATTCCAGGGGGCTCGT
         F  L  R  T  C  S  S  Q  W  P  S  V  S  D  H  S  R  G  L  V
601    GATAACTGCCAAGGAAGGAGGGACAAGCATTGATTCATCGGCCTCGAGTAGCCTTCGATG
         I  T  A  K  E  G  G  T  S  I  D  S  S  A  S  S  S  L  R  C
661    CCTCTCTTCCATCGTGGACAGCATTTCCTCGGAGGAACACAAACTCCCCTGCGTGGAGGA
         L  S  S  I  V  D  S  I  S  S  E  E  H  K  L  P  C  V  E  E
721    GGTGGTGGAGAAGTAACTCAGTGGTCCGGACGTTCTCCACGGAGCAGGAAGATC
         V  V  E  K  *
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Fig. 3. *The nucleotide sequence and the deduced amino acid sequence of Jiulong yak Myf6*

The primers used for the cloning of *Myf6* gene were shaded. An asterisk represents the stop codon. The Basic domain of Jiulong yak *Myf6* was underline; The Helix-loop-helix(HLH) domain was boxed.

Table 1. *List of the Myf6 sequences used in the analyses*

Organism	GenBank ID	Amino acid identity(%)
Bos grunniens	AGF90517.1	
Bos taurus	NP_861527.1	100
Capra hircus	NP_001272531.1	99
Ovis aries	NP_001128254.1	99
Sus scrofa	NP_001231601.1	98
Homo sapiens	NP_002460.1	98
Ailuropoda melanoleuca	XP_002916823.1	98
Equus caballus	XP_001490030.1	97
Callithrix jacchus	XP_002752845.1	97
Oryctolagus cuniculus	XP_002711410.1	97
Rattus norvegicus	NP_037304.1	95
Mus musculus	NP_032683.1	94
Canis lupus familiaris	XP_003432065.1	93
Anas platyrhynchos	XP_005010861.1	92
Monodelphis domestica	XP_001372463.2	88
Chelonia mydas	EMP36510.1	87
Gallus gallus	NP_001025917.1	86
Taeniopygia guttata	XP_002195106.1	85
Ornithorhynchus anatinus	XP_001505391.1	84
Xenopus laevis	NP_001088572.1	75
Danio rerio	NP_001003982.1	60
Ctenopharyngodon idella	AFL56777.1	61
Salmo salar	NP_001117079.1	63
Takifugu rubripes	CAC39207.1	61

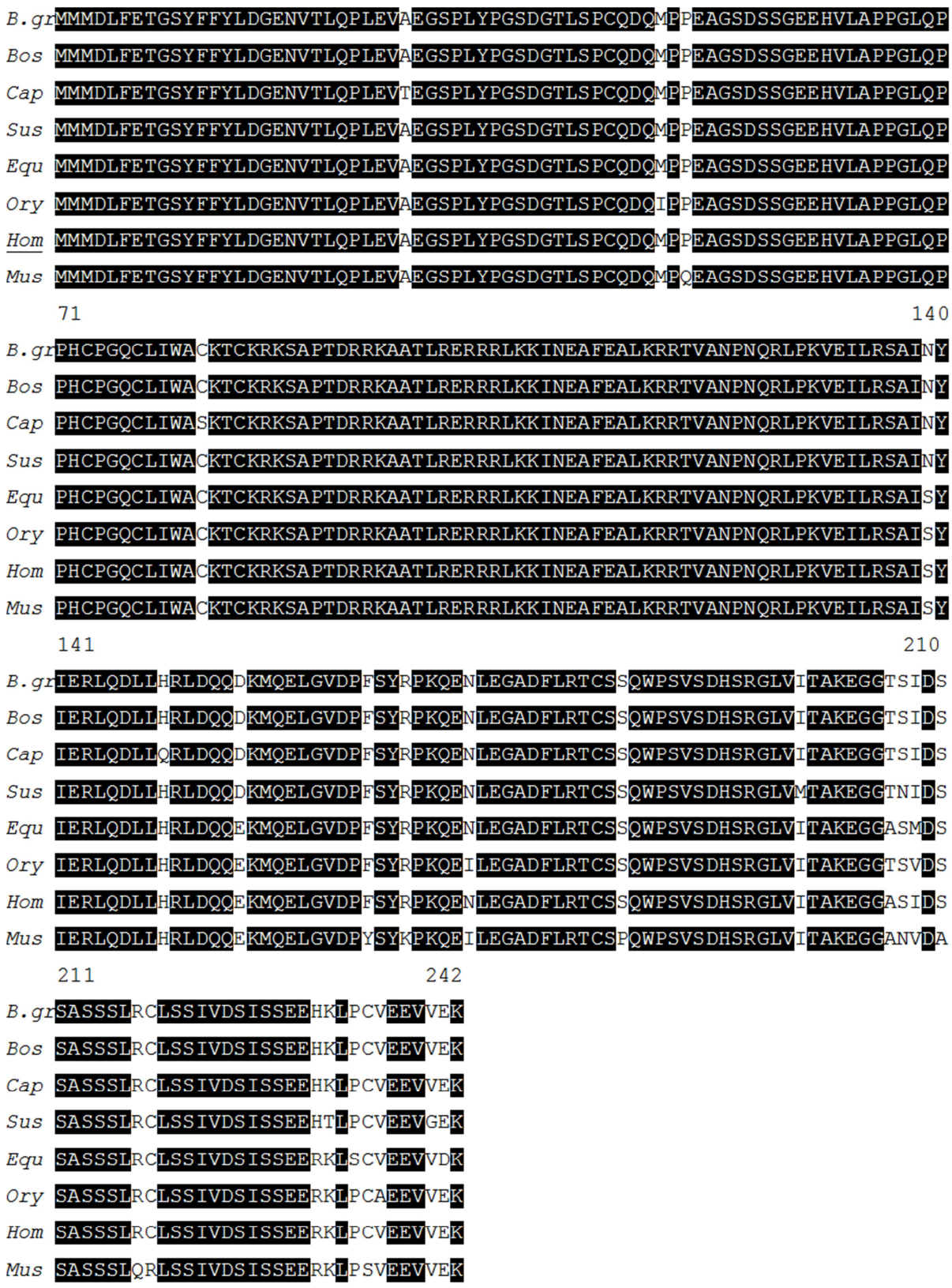
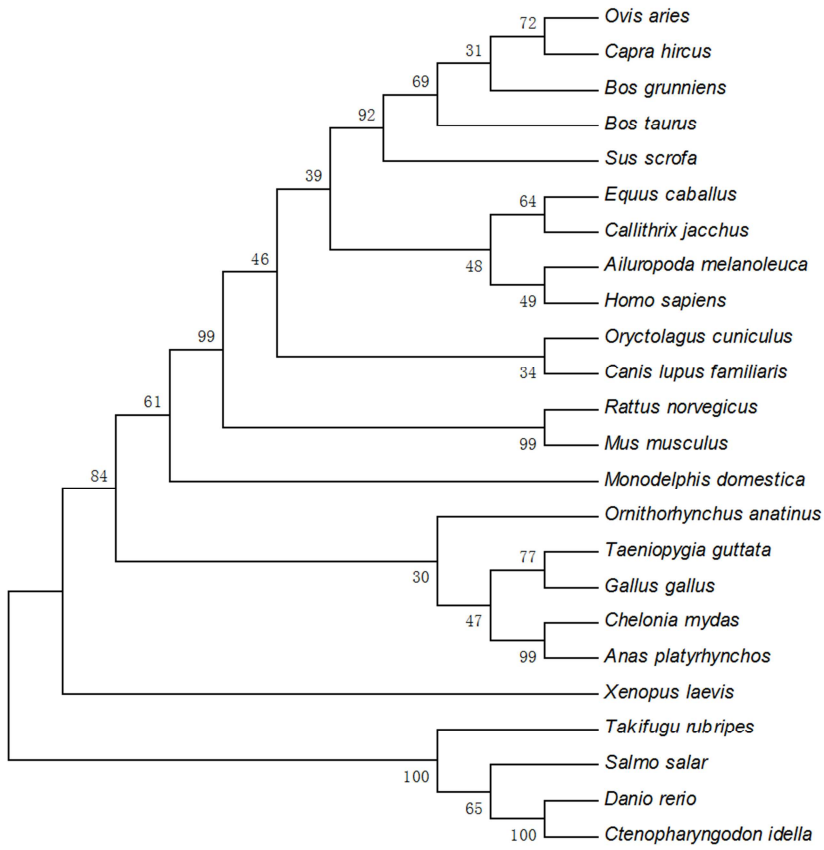


Fig. 4 (a). *Mulit-alignment of the Myf6 of eight species*

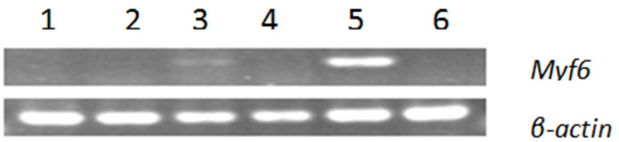


The GenBank accession numbers of the Myf6 sequences are listed in Table 1

Fig.4 (b). *Phylogenetic tree of Myf6 of various species*

Multiple sequence alignments of the deduced protein sequences of *Bos grunniens* Myf6 with other Myf6 sequences were shown in Fig.4a. *Bos grunniens* Myf6 was 100% identical to *Bos taurus*, 99% to *Capra hircus*, 98% to *Sus scrofa* and *Homo sapiens*, 97% to *Equus caballus* and *Lepus sinensis*, and 96% to *Mus musculus*. A phylogenetic tree to assess the relationship of yak Myf6 with other known Myf6 was performed (Fig. 4b). The Myf6 protein largely clustered into two major groups. The yak Myf6 grouped together with cattle as the closest neighbor, apart from *Actinopterygii* Myf6.

In male adult yaks (3.5-5.5 yr, n=5), the highest level of *Myf*6 mRNA was observed in *Longissimus* muscles among tissues examined ($p<0.05$), and trace expression in spleen. But *Myf*6 mRNA expression could not be detected in heart, liver, kidney, and adipose tissues (Fig. 5). Expressions of *Myf*6 gene in *Longissimus dorsi* of male yaks increased with age. *Longissimus dorsi* of 0.5 yr yaks contained significantly lower level of *Myf*6 mRNA than those of 3.5-5.5 yr and over 9.0 yr yaks ($p < 0.05$)(Fig. 6).



1, heart; 2, liver; 3, spleen; 4, kidney; 5, *longis simus* muscle; 6, fat

Fig. 5. *RT-PCR result of the Myf6 expression in Jiulong yak tissues*

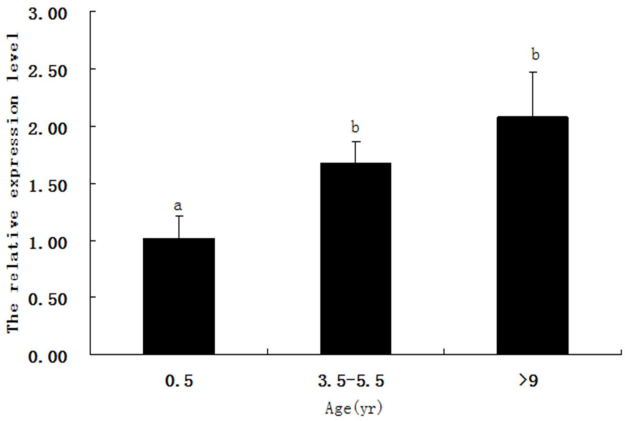


Fig. 6. *The abundance of Myf6 mRNA in longissimus muscle of Jiulong yaks at different ages*

4. Discussion

The muscle expression of particular genes such as myogenic transcription factors could significantly affect the meat content in carcass, as well as meat quality. Therefore, analysis of genes transcription would provide valuable information about genetic background of leanness and meat quality, what is essential for meat production. Myogenesis is mainly controlled by genes of MRF family, which encodes the basic helix-loop-helix proteins that initiate the formation of muscle fiber and regulate the transcription of muscle specific

genes [22]. Expressions and interaction of these factors of MRF family form a center of regulating network, which affect myogenesis by including the cascade expression of other muscle-specific genes and participating in precise regulation of the balance between proliferation and differentiation of primary muscle cells[23-26]. As an important member of MRF family, Myf6 is involved in the processes of differentiation and maturation of myotubes during embryogenesis and continues on a relatively higher level than the other myogenic regulatory factors in adult muscle, affecting muscle fiber phenotype and maintaining the differentiated phenotype [12-14]. This gene has 10-fold higher postnatal expression than the other genes of the MRF family (Bober et al. 1991)[27]. Increases in *Myf*6 mRNA or protein could affect muscle fiber size and number [28,29], prompt the mRNA transcription of heavy chains of three subtype of myosin, and result in muscular hypertrophy[30].

The expression profiles of *Myf*6 gene in skeletal muscle were evaluated in growing young gilts of different breeds at different ages [31,32]. The breed-specific and age-dependent expression profiles analysis revealed highly significant ($p<0.01$) differences in *Myf*6 expression levels of all skeletal muscles among investigated breeds and no significant relationship between gilts ages and the expression levels of *Myf*6 gene. These two studies found the expression of *Myf*6 gene in skeletal muscle of Pietrain was higher than in gilts of other breeds, which may suggest that higher *Myf*6 gene expression would be related to higher muscularity of carcass, as it is in Pietrain breed. In this study, we found significant ($p<0.05$) differences between ages and the expression levels of *Myf*6 gene in *Longissimus* muscles of yak. Expression of *Myf*6 gene in *Longissimus dorsi* of male yaks increased with age. The levels of *Myf*6 mRNA in *Longissimus* muscles of 3.5-5.5 yr and over 9.0 yr yaks were significantly higher than that of 0.5 yr yaks ($p<0.05$).

Interestingly, extensive studies revealed that Myf6 tends to be expressed more highly in muscle tissue of the lean selection line which has higher lean mass and expressions of Myf6 in the thicker muscle fibers for maintenance [15,31,32]. Therefore, *Myf*6 is supposed to be one promising candidate gene for growth- and meat quality-related traits in pig and cattle [17,33,34]. To our knowledge, this study is one of the first analyses of postnatal expression of *Myf*6 gene in yak. Significant differences between *Myf*6 expression and ages allowed us to select this gene for further trait-associated studies. The further identification of casual polymorphism and determination of functional role are even more challenging, since there are many different molecular mechanisms through which expression activity of specific genes in myogenic cells can be regulated.

Acknowledgements

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