Gene 656 (2018) 9–16

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Polymorphism in promoter of *SIX4* gene shows association with its transcription and body measurement traits in Qinchuan cattle

Dawei Wei[a,b,1], Sayed Haidar Abbas Raza[a,b,1], Jiupan Zhang[c], Linsheng Gui[a], Siddiq Ur Rahman[d], Rajwali Khan[a], Seyed Mahdi Hosseini[e], Hubdar Ali Kaleri[f], Linsen Zan[a,b,*]

[a] College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China
[b] National Beef Cattle Improvement Center of Northwest A&F University, Yangling 712100, China
[c] Guyuan Branch of Ningxia Academy of Agriculture and Forestry Sciences, 756000 Guyuan, China
[d] College of Life Sciences, Northwest A&F University, 712100 Yangling, China
[e] College of Animal Science and Technology, Huazhaong Agricultural University, Wuhan, Hubei 430070, China
[f] Department of Animal Science and Aquaculture, Dalhousie University, Canada

ARTICLE INFO

Keywords:
SIX4 gene promoter
SNPs
Body measurement traits
Qinchuan cattle

ABSTRACT

The sine oculis homeobox homolog 4 (*SIX4*) gene belongs to the SIX gene family, which plays a critical role in muscle regeneration and early stages of ontogeny. This study aimed to detect promoter variations of bovine *SIX4* genes in Qinchuan cattle, and to evaluate the effect of transcription regulations and body measurement traits. Quantitative real-time PCR (qPCR) results showed that the mRNA expression levels of *SIX4* gene were found significantly highest in *longissimus thoracis* tissue and individual before attaining the stage of physiological maturity. Using sequencing technology on a total of 428 Qinchuan cattle, seven single nucleotide polymorphisms (SNPs) were identified in the promoter region of *SIX4*, and seven haplotypes representing 18 potential transcription factor compositions of polymorphic potential *cis*-acting elements. Association analysis indicated that the H_3-H_3 diplotype performed greater withers height, chest depth, chest circumference, back fat thickness and ultrasound loin muscle area ($P < 0.05$) than H_5-H_6, which were consistent with the promoter activity of Hap3 haplotype was higher than the Hap5 and Hap6 haplotype in vitro. These potential transcription factor information and combined genotypes H_3-H_3 of the *SIX4* gene can be used as a molecular marker for selection of economic traits in Qinchuan cattle.

1. Introduction

China cattle beef industry is growing rapidly to meet the meat demand of large population. The Qinchuan breed of cattle (*Bos taurus*) is an indigenous beef breed having peculiar qualities and physical features. This breed is utilized widely in beef production in China and elsewhere. However, Qinchuan cattle exhibit a number of limitations compared to imported commercial beef cattle breeds, such as slow growth rate and underdeveloped hind hips. Therefore, these traits have been recognized as primary goals for genetic improvement of the Qinchuan breed. Traditional methods of breed improvement through breeding and selection in large ruminants such as cattle needs decades and still very difficult to be achieved due to long generation interval and low fertility rate. Rapid progress depends on the identification of reliable molecular markers linked with trait(s) of interests Quantitative trait loci (QTL) analyses have shown that body measurement traits (BMTs) are quantitative traits controlled by numerous genes with only minor individual effects (Boucher et al., 1996). The identification of statistically significant associations between genetic variants within candidate genes provides potentially powerful approach to accelerate breeding efforts with these traits for Qinchuan cattle breed improvement (Hirwa et al., 2011).

The Drosophila sine oculis (*so*) locus gene plays an essential role in patterning the eye imaginal disk In mammals, the *so* gene family, designated *SIX*, consist of six members designated as *SIX1* to *SIX6* and Six proteins are characterized by presence of a Six domain (SD) and Six-

Abbreviations: qPCR, Quantitative real-time PCR; SNPs, single nucleotide polymorphisms; QTL, Quantitative trait loci; BMTs, body measurement traits; SD, Six domain; HD, Six-type homeodomain; MAS, Marker assistant selection; BL, body length; WH, withers height; CD, chest depth; CC, chest circumference; ULA, loin muscle area; BF, back fat thickness; IFC, intramuscular fat content; PIC, polymorphism information content; LD, linkage disequalibrium; GLM, general linear models

* Corresponding author at: College of Animal Science and Technology, Northwest A&F University, 712100 Yangling, China.
E-mail address: zanlinsen@163.com (L. Zan).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.gene.2018.02.059
Received 3 January 2018; Received in revised form 14 February 2018; Accepted 23 February 2018
Available online 26 February 2018
0378-1119/

type homeodomain (HD) (Kawakami et al., 2000), which confer specific DNA binding activity and transcription factor function (Liu et al., 2010). Among the *SIX* gene family members, *SIX1* and *SIX4* are known to play important roles in myogenesis, and expression of *SIX1* and *SIX4* is readily detectable in myogenic cells in the precursor of trunk musculature, particularly in muscle precursors of the developing limb buds (Ozaki et al., 2001) In addition, *SIX4* participates in myogenesis during muscle regeneration through enhancing expression of *MyoD* (Relaix et al., 2013). Also, *SIX4* and Eya cooperatively regulate transcription from the myogenin promoter through direct binding to the MEF3 site in cultured cells (Liu et al., 2010).

Furthermore, *SIX1* and *SIX4* drive the transformation of slow-twitch toward fast-twitch (glycolytic) fate during myogenesis in adult mouse muscle (Grifone et al., 2004). Slow-twitch and fast-twitch muscles fibers are principal factors influencing muscles characteristics and meat tenderness (Renand et al., 2001). Taken together, these data indicate that *SIX4* is critical for skeletal myogenesis and skeleton muscle development.

Previous studies have demonstrated that *SIX4* gene function appears to be closely associated with muscle development. In addition, G. Wang et al. (2014) has reported that three single nucleotide polymorphisms (SNPs) exist in the bovine *SIX4* exon 2 and 3 were significantly associated with BMTs. For gene promoter region variants influence the enzyme activity by altering the gene expression and transcriptional activity, thereby affecting the individual development (Pastinen and Hudson, 2004). Hence, we hypothesized that the promoter region variants of *SIX4* gene might influence transcriptional regulation and association with its BMTs in Qinchuan cattle. Here in, the objectives of this study were to identify the genetic polymorphisms at the 5′UTR of the bovine *SIX4* gene and analyze the effect of transcriptional activities that are associated with BMTs in Qinchuan cattle. The knowledge of *SIX4* obtained in this study may contribute to further understanding of the roles of *SIX4* in myogenesis and provide a new tool for BMTs.

2. Materials and methods

2.1. DNA samples and data collections

This study was performed with a total of 428 female cows (aged 18 to 24 months, and unrelated for at least three generations) randomly selected from the Experimental Farm of National Beef Cattle Improvement Center's experimental farm in Yangling, China. And for this trial, the blood samples of trail animals were collected from the population treated in the condition as coincident as possible, to reduce the background error. They were fed with the same of roughage to concentrate ratio (6:4), in the similar rearing environment (similar temperature, humidly etc.), and in alike management process. BMTs, including body length (BL), withers height (WH), chest depth (CD), chest circumference (CC) were measured as described previously (Gui et al., 2015). Back fat thickness (BF), ultrasound loin muscle area (ULA) and intramuscular fat content (IFC) were measured by ultrasound using Sono-grader (Renco, USA) according to the manufacturer's protocol. Briefly, the measurement parts of BF, ULA and IFC between the numbers 12 to 13 of ribs. Place ultrasonic probe within 5 cm of the spinal and vertical alignment. The data of BF, ULA and IFC were collected by system program. Genomic DNA was extracted from blood samples using a standard method phenol-chloroform protocol (Sambrook and Russell, 2001).

2.2. Quantitative PCR analysis of SIX4 gene expression patterns

Fourteen tissues (heart, liver, spleen, lung, kidney, rumen, reticulum, omasum, abomasum, small intestine, large intestine, abdominal fat, *longissimus thoracis* and testicular tissue) were obtained from three two-year-old bulls of Qinchuan cattle. The *longissimus thoracis* samples were collected from eight developmental stages of male Qinchuan cattle, including 1, 6, 9, 12, 18, 24, 36 and 60 months after birth, and three parallel individuals for each period. Total RNA was extracted using the Total RNA kit (TaKaRa, Dalian, China) and cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa). RT-PCR reaction mixtures (20 μL) contained SYBR Green Real-time PCR Master Mix (TaKaRa), gene-specific primers (Table S1) and template cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (*β-actin*) were used as endogenous control gene. The cycling conditions consisted of an initial 5 min at 95 °C, 34 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Reactions were run in triplicate using a 7500 System SDS V 1.4.0 thermocycler (Applied Biosystems, USA). The relative expression levels of the target mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3. SNP detection and genotyping

Two pairs of PCR primers (primer A and B) were designed to amplify a ~1.3 kb promoter region, up to and including the translational start site, of the bovine *SIX4* gene (NCBI accession AC_000167, from 73124222 to 73125547). The primer sequences are shown in Table S1. Firstly, we detected the mutations of the *SIX4* gene by using the 428 individual DNA samples were mixed with equal molar ratio to form a DNA pool (Sham et al., 2002). Then, PCR amplifications were performed using 428 Qinchuan cattle individual DNA. PCR reactions (50 μL) contained 100 ng of pooled genomic DNA (individual DNA), 1 μM of primer, 1 × buffer (including 1.2 mM $MgCl_2$), 400 μM dNTPs, and 0.8 units of KOD DNA Polymerase (Toyobo, Osaka, Japan). All PCR products were sequenced to verify amplification of the intended target. Finally, the sequences were imported into BioXM software (Version 2.6) for SNP analysis.

2.4. Potential cis-acting elements identification

Potential *cis*-acting elements with variations located in or adjacent to their recognition sequences within the ~1.3 kb *SIX4* promoter were identified using the Genomatix database and web server (http://www.genomatix.de).

2.5. Cell culture and transfection

C2C12 myoblast cells were maintained in DMEM Medium supplemented with 10% fetal bovine serum (FBS) (NBCS; Invitrogen, USA),100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2 in an atmospheric incubator. Cells were grown in 24-well plates, overnight to 80–90% confluence at a density of 1.2×10^5 cells.

The fragment of 1376 bp of the promoter ranged from −1326 bp to +50 bp harbored different haplotypes were generated by specific primers with the sequence of the *Xho*I and *Hind*III restriction sites (Table S1). Amplification products were cloned into vector pMD19-T (TaKaRa) and ligated into the *Xho*I and *Hind*III sites of the luciferase reporter vector pGL3-basic digested with the same restriction enzymes *Xho*I and *Hind*III (TaKaRa). These plasmids were named pGL3-Haps. The reporter plasmid was co-transfected with plasmid Renilla luciferase reporter plasmid (Promega, USA) into C2C12 myoblast cells with X-tremeGENE HP DNA transfection reagent (Roche, USA). Firefly luciferase activity and Renilla luciferase activity were measured according to the dual-luciferase reporter assay standard protocol in three independent experiments. Luciferase activity was measured using the Dual Reporter assay system (Promega Corp) and NanoQuantPlate™ (TECAN, infinite M200PRO)·The level of firefly luciferase activity was normalized to Renilla luciferase activity and expressed as arbitrary units.

2.6. Data analyses

Allelic frequencies, genotype frequencies, observed and expected