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Novel polymorphisms of *SIX4* gene and their association with body measurement traits in *Qinchuan* cattle

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ABSTRACT

Sine oculis homeobox homolog 4 (*SIX4*) gene belongs to the *sine oculis*/*SIX* gene family, which includes six members in vertebrates. *SIX4* gene plays a crucial role in skeletal myogenesis, and its genetic variations or deficiency may cause hypopituitarism, suggesting that *SIX4* gene is a potential candidate gene affecting body measurement traits (BMTs) in animals. Herein, the objectives of this study were to identify genetic polymorphisms of bovine *SIX4* gene and to analyze potential association between single nucleotide polymorphisms (SNPs) and body measurement traits in *Qinchuan* cattle. In the present study, we investigated polymorphisms of *SIX4* gene in 426 *Qinchuan* cattle using DNA sequencing and polymerase chain reaction–restriction fragment length polymorphisms. Three novel SNPs were identified within bovine *SIX4* gene. Associations between body measurement traits and *SIX4* gene polymorphisms were investigated, and significant statistical associations were found between polymorphisms of these three SNPs and body measurement traits ($P < 0.05$). Hence, based on results obtained from this study, we conjectured that *SIX4* gene may have potential effects on body measurement traits in *Qinchuan* cattle population and could be used for marker-assisted selection.

1. Introduction

The economic importance of body measurement traits (BMTs) has aroused increasingly attention in cattle selection and breeding. As we know, it is difficult to improve body measurement traits by traditional breeding methods. However, application of DNA markers for improving these traits through marker-assisted selection (MAS) is a powerful and efficient strategy (Pedersen et al., 2009). Numerous researches have indicated that correlation did exist between genotype and BMT variation, suggesting that genetic factors are predominant. Consequently, it would be reasonable to place considerable emphasis on sifting out numerous candidate genes and elaborating the significant associations between their genetic variations with BMTs.

The mammalian *SIX* family genes, homologues of *Drosophila sine oculis*, consist of six members, *SIX1* to 6 (Kawakami et al., 2000). The family proteins are characterized by the presence of the *SIX* domain and *SIX*-type homeodomain, which confer specific DNA binding activity and function as transcription factors (Xu, 2013; Relaix et al., 2013). During skeletal muscle development, both *SIX1* and *SIX4* were expressed in the somite and migrating myoblasts and play important roles in myogenesis (Grifone et al., 2004; Grifone et al., 2005; Niro et al., 2010). In addition, *SIX1* and *SIX4* are necessary for the induction of the fast-type-muscle program during myogenesis (Niro et al., 2010) and are involved in the assignment of the fast/glycolytic character of the myofiber in adult skeletal muscle (Grifone et al., 2004). Moreover, *SIX*/*SIX4* double KO mice showed an aggravation of the phenotype for the single *SIX1* KO (Grifone et al., 2005). Besides, in cotransfection studies, *SIX4* transactivated the MCK enhancer as well as muscle-specific regulatory regions of Aldolase A and Cardiac troponin C (Himeda et al., 2004). Furthermore, *SIX4* activated the *myogenin* gene promoter alone or in synergy with the specific cofactor, *Eya*, through direct binding to the MEF3 site in cultured cells (Xu, 2013).

The identification of genetic markers associated with important economical traits of livestock species has the potential to alter the genetic improvement rate via marker-assisted selection (MAS) (Nkrumah et al., 2004). Previous studies have demonstrated that *SIX4* expression appeared to be closely related to muscle development and might influence the growth development of mammals if genetic mutations of *SIX4* occurred. However, the association of *SIX4* gene variations with body measurement traits in cattle has not been reported yet. Hence, we proposed the hypothesis that the polymorphisms of the *SIX4* gene were

Abbreviations: BMTs, Body measurement traits; SNPs, Single nucleotide polymorphisms; PCR, Polymerase chain reaction; RFLP, Restriction fragment length polymorphisms; MAS, Marker-assisted selection; BL, Body length; WH, Withers height; HH, Hip height; RL, Rump length; HW, Hip width; CD, Chest depth; CC, Chest circumference; PBW, Pin bone width; He, Gene heterozygosity; Ne, Effective allele numbers; PIC, Polymorphism information content; dNTP, Deoxyribonucleoside triphosphate; UV, Ultraviolet.

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significantly associated with body measurement traits. The results obtained could provide essential genetic information to identify and efficiently utilize bovine genetic resources through MAS program.

2. Materials and methods

2.1. Resource populations, data collection and DNA samples

The 426 *Qinchuan* cattle from one experimental farm were randomly selected to be unrelated for at least three generations from breeding population, with the aim of having diverse lineages. The traits, including body length (BL), withers height (WH), hip height (HH), rump length (RL), hip width (HW), chest depth (CD), chest circumference (CC) and pin bone width (PBW), were assigned to be measured by the same person as described previously (Ozkaya and Bozkurt, 2009) only to minimize systematic error. DNAs were extracted from blood obtained from the jugular vein of 426 *Qinchuan* cattle following the protocol of Sambrook and Russell (2001). The final DNA pellets were stored at 4 °C for immediate use while at −20 °C for long term.

2.2. PCR amplification and sequencing

Based on the nucleotide sequence of the bovine *SIX4* gene (GenBank accession number AC_000167), two following primers were designed to amplify the major part of the coding region of the *SIX4* gene with Premier 5.0 software. P1 (F: 5′ GGGACCCAATGGAGTTAT 3′ and R: 5′ AAATAAAAGCCCACCCC 3′) was used to amplify 659 bp PCR product for exon 2; P2 (F: 5′ GCCTGGAAATCTGTAGTTGTA 3′ and R: 5′ CAGTTC TGAAGAGCTGCATAG 3′) was used to amplify 568 bp PCR product for exon 3. Each amplification reaction was carried out in a 15 μL reaction mixture containing 30 ng genomic DNA, 0.4 μM of each primer, 7.5 μL 2× Reaction Mix (500 μM dNTP each, 20 mM Tris–HCl (pH 8.3) 100 mM KCl, 3 mM $MgCl_2$, other stabilizer and enhancer), and 0.30 U Golden DNA polymerase (Tiangen Biotech, Beijing, China). The amplifications were carried out in thermal cycler (Eppendorf, Germany) with the following conditions: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s, extension at 72 °C for 30 s and lastly the final extension of 10 min at 72 °C. Amplification was verified by electrophoresis of products in 1% (w/v) agarose gel in 1× TBE buffer, parallel with 2000 bp DNA marker (Fermentas) as a molecular weight marker for confirmation of the length of PCR products. Gels were stained with etidium bromide (10 mg/mL) and amplification products were visualized and documented as exposed to UV light. After PCR amplification (Ma et al., 2012), the PCR products were sequenced in both directions in an ABI PRIZM 377 DNA sequencer (Perkin-Elmer) directly for identification of SNPs. The sequence maps were analyzed with SeqMan software.

2.3. Genotyping of SIX4 allele by PCR–RFLP

Restriction fragment length polymorphism (RFLP) analyses were used to identify the genotypes of SNPs. The PCR products were digested in a total volume of 10 μL containing: 5 μ L of PCR product, three units (0.3 μL) of restriction enzyme(*Tai*I, *Tsp*45I and *Hinf*I), 1 μL of reaction buffer and 3.7 μL of ddH_2O. The mixture was incubated for 16 h at 37 °C. The digested products were then detected by electrophoresis in 1.5% agarose gel stained with ethidium bromide. To confirm the results based on the PCR–RFLP technique, the PCR products of different electrophoresis patterns were sequenced in both directions.

2.4. Statistical analysis

The following items, including genotypic frequencies, allelic frequencies, Hardy–Weinberg equilibriums, gene heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) were statistically analyzed according to the previous approaches of Nei and Roychoudhury (1974) and Nei and Li (1979). SHEsis software was used to perform haplotype analysis (Li et al., 2009).

SPSS software (version 17.0, USA) was carried out to analyze associations between SNPs and BMTs. Statistical analysis was performed on the basis of body measurement records (including BL, WH, HH, RL, HW, CD, CC and PBW) according to the following statistical linear model:

$$Y_{ijk} = \mu + G_j + A_i + E_{ijk} \quad (1)$$

where Y_{ijk} is the observation for the BMTs, μ is the overall mean for each trait, G_j is the genotype effect, A_i is the fixed effect of age and E_{ijk} is the random error for the ijkth individual (Li et al., 2013).

3. Results and discussion

3.1. Analysis of polymorphisms of the candidate gene SIX4 in Qinchuan cattle population

The bovine *SIX4* gene is located on chromosome 10 and has 3 exons encoding 568 amino acids. In the present study, three variations (AC_000167: g.1726C>T, g.1886G>T, g. 5456G>A) including one transversion and two conversions were revealed in exon 2 and exon 3 by comparing the sequencing results with the DNA sequence of Bos taurus *SIX4* gene published in GenBank (Fig. 1). PCR–RFLP was used to further genotype the individuals. According to the sequence mutations, the PCR products could be digested with *Tai*I, *Tsp*45I and *Hinf*I restriction enzymes to genotype the individuals respectively. Interestingly, it was originally discovered that these three novel mutations could be detected by endonucleases restriction site in bovine *SIX4* gene.

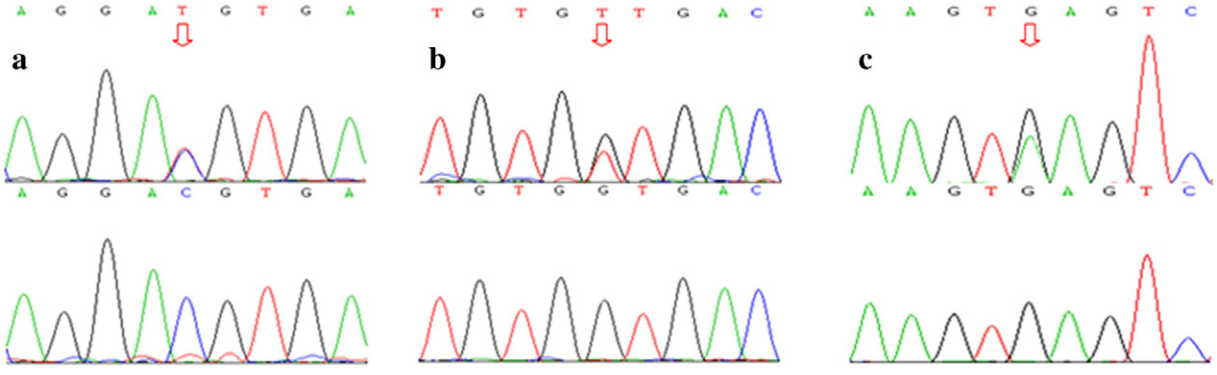

Fig. 1. The sequencing results of the bovine *SIX4* gene and three SNPs were indicated by red arrows. Note: a: g.1726C>T b: g.1886G>T c: g.5456G>A.