Gene 669 (2018) 1–7

Contents lists available at ScienceDirect

Gene

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

Research paper

Bovine pituitary homeobox 2 (*PITX2*): mRNA expression profiles of different alternatively spliced variants and association analyses with growth traits

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ARTICLE INFO

Keywords:
PITX2
Cattle
Deletion
Alternative splicing
Expression pattern

ABSTRACT

Pituitary homeobox 2 (*PITX2*) plays crucial roles in embryogenesis, ontogenesis, growth, and development *via* the Wnt/beta-catenin and POU1F1 pathways. To better understand the characteristics and genetic effects of the cattle *PITX2* gene, we identified alternative *PITX2* splicings, examined the effects of the spliced variants on mRNA expression levels in tissues, and then used association analyses to explore the relationships between a *PITX2* deletion genetic variant and growth traits in 750 native Chinese cattle. An unreported spliced variant of *PITX2*, designated here as *PITX2-V1*, was identified in cattle using *in silico* cloning and RT-PCR. The entire coding sequence of *PITX2* is 978 bp, encoding 325 amino acids, whereas that of *PITX2-V1* is 357 bp encoding 118 amino acids. Cattle *PITX2* exhibited both a perfect homeodomain and an OAR domain, but *PITX2-V1* lacked the homeodomain. Analyses with qRT-PCR showed that the expression level of *PITX2* in cattle testis was very low, and *PITX2-V1* was only very slightly expressed in the brain and testis. Furthermore, a 24 bp deletion was detected within *PITX2* intron, and the different genotypes were significantly associated with growth traits (*e.g.*, body height, body length, heart girth) in four cattle breeds ($P < 0.05$). These results are of direct benefit to future cattle breeding, and provide new insights into the characteristics and functions of cattle *PITX2* gene.

1. Introduction

Pituitary homeobox 2 (*PITX2*) was first identified in human patients with Axenfeld Rieger syndrome, a craniofacial disorder, and is thus commonly referred to as the *RIEC* gene (Semina et al., 1996). *PITX2* is a member of the PITX (paired-like homeodomain transcription factor) family, having a paired-like homeodomain (HOX) and an OAR domain. The two domains are important for the binding capacity of the PITX2 protein and the DNA sequence (Amendt et al., 1999). *PITX2* is widely expressed in a variety of tissues and is conserved across multiple species, implying its general importance and the many physiological functions it plays in organisms (Shih et al., 2007). Previous studies have reported that *PITX2* plays roles in embryogenesis, vertebrate left-right asymmetry development, cell proliferation, cell differentiation, and myogenesis through the Wnt/beta-catenin pathway (L'honoré et al., 2016; Hernandez-Torres et al., 2017). Moreover, *PITX2* is involved in the POU1F1 pathway, which is strongly associated with mammalian lactation, growth, and development (Davis et al., 2010; Zhao et al., 2013). However, the functions of the *PITX2* gene in cattle remain unclear.

Alternative splicing (AS) have received increasing attention over recent years. AS occurs on the precursor mRNA during gene expression, resulting in a single gene producing multiple spliced variants and generating functionally diverse proteins (Li et al., 2013; M. Zhang et al., 2015). AS is important in mediating gene expression, and thus is useful in the analyses of evolutionary pathways, metabolic activity, and organismal complexity, among others (Zhou et al., 2014). For example, the different transcripts produced by the transcription factor MEF2 family genes have diverse functions in regulating myogenesis; *MEF2Cα1* is a ubiquitously expressed isoform that exhibits no myogenic activity, whereas *MEF2Cα2* is a muscle-specific *MEF2C* isoform required for efficient tissue differentiation (S. Zhang et al., 2015a).

Abbreviations: SNP, single nucleotide polymorphism; *CNV*, copy number variation; *PITX2*, pituitary homeobox 2; *PITX*, paired-like homeodomain transcription factor; *PITX2-V1*, PITX2-variant 1; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *NCBI*, National Center of Biotechnology Information; *LEF1*, lymphoid enhancer-binding factor 1; *LEFTY1*, left-right determination factor 1 precursor; *LEFTY2*, left-right determination factor 2 precursor; *ARX*, Aristaless-related homeobox; *aa*, amino acids; *HOX*, homeodomain; *AS*, alternative splicing; *MAS*, marker-assisted selection; *HWE*, Hardy-Weinberg equilibrium; *He*, heterozygosity; *Ne*, effective allele numbers; *PIC*, polymorphism information content

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https://doi.org/10.1016/j.gene.2018.05.083
Received 7 February 2018; Received in revised form 12 May 2018; Accepted 21 May 2018
Available online 22 May 2018
0378-1119/

Different spliced variants of *PITX2* have been identified in a variety of species except cattle. For example, three kinds of isoforms have been identified in humans, specifically *Pitx2a*, *Pitx2b*, and *Pitx2c*. Differences among these isoforms occur primarily on the N-end of the proteins (Arakawa et al., 1998). In *zebrafish*, three isoforms have been identified, and different isoforms were found to have different functions: *Pitx2a* regulates zebrafish heart development, whereas *Pitx2c* is associated with the development of *zebrafish* brain and intestinal tract (Schweickert et al., 2000). Five and three isoforms of *PITX2* have been identified in *Mus musculus* and goats, respectively, the expression of which was determined to be tissue-specific (Liu et al., 2003; Zhang et al., 2016). To the best of our knowledge, however, no research has been conducted on *PITX2* alternative splicing in cattle.

Single nucleotide polymorphism (SNP), short sequence insertion/deletion, copy number variation (CNV), and other DNA genetic variations have come under increasing focus in gene function research (Julienne et al., 2010). SNPs are commonly used DNA molecular markers in marker-assisted selection (MAS), but identifying polymorphisms of a single nucleotide is difficult (Zhao et al., 2013). CNV is a type of long segment (length > 50 nt) duplication or deletion event that can be identified with qRT-PCR method, but this approach incurs far greater cost than traditional PCR (Sharp et al., 2005). Insertion/deletion, being a kind of DNA short segment genetic variation, has greater influence on gene function than SNP, and the detecting methods of insertion/deletion is more convenient and practical than SNP and CNV (Fan et al., 2007). However, the functions of short sequence insertion/deletion variants within the *PITX2* gene in cattle have not yet been thoroughly explored.

In this study, we first identified the novel spliced variants of cattle *PITX2* gene using *in silico* cloning and experimental methods, following which we analysed the characteristics of the different spliced variants and their effects on mRNA expression levels. In addition, we assessed the effects of a short sequence deletion variant of the *PITX2* gene on cattle growth traits. We believe that such research would enrich the understanding of the functions of the *PITX2* gene in cattle and consequently improve MAS-based cattle breeding.

2. Materials and methods

2.1. Ethics statement

Experimental animals and procedures used in this study were approved by the Faculty Animal Policy and Welfare Committee of Northwest A&F University (NWSUAF) under contract. The care and use of experimental animals fully complied with local animal welfare laws, guidelines, and policies.

2.2. Sample and data collection

Heart, liver, spleen, lung, kidney, muscle, brain, ovary, and testis tissue samples were collected from three Qinchuan cattle foetuses (one females and two males) obtained from a local farm in Shaanxi Province, China. All tissue samples were snap-frozen in liquid nitrogen and subsequently stored at −80 °C until RNA isolation (Zhou et al., 2014). In addition, 750 genomic DNA samples were collected from five Chinese beef cattle breeds: Nanyang (n = 78), Luxi (n = 30), Qinchuan (n = 303), Pi'nan (n = 128), Ji'an (n = 211), and these cattle breeds were reared in the provinces of Henan, Shandong, Shaanxi, Jiangxi, Henan, respectively. All of these animals were healthy, unrelated (through at least three generations), 2–3 yr of age (except Nanyang individuals) non-pregnant cows. Growth traits of all individuals were measured by farm staff at their respective farms in accordance with Gilbert's method, and measurements were taken when the cows were standing naturally on even ground (Gilbert et al., 1993). Nanyang individuals were fed a mix of concentrate and straw diet *ad libitum* after weaning at 6 months old; the measured growth traits of Nanyang cattle included body weight, body height, body length, heart girth, hucklebone width, birth weight, and average daily gain of 6, 12, 18, and 24 months of age (Sun et al., 2013). The measured growth traits of Luxi cattle included body weight, body height, body length, heart girth, and cannon circumference. Qinchuan individuals were raised on a corn-corn silage diet after weaning at ~6 months, and were fed *ad libitum* under normal conditions. The measured growth traits of Qinchuan and Pi'nan cattle included body weight, body length, body height, heart girth, rump length, hucklebone width, height at hip cross, and hip width; chest width and chest depth of Qinchuan cattle were also measured (Dang et al., 2014; S. Zhang et al., 2015b; Jin et al., 2016). The growth traits of Ji'an cattle individuals were not measured. Besides, growth indices were calculated from the data obtained from the measured body traits, and the calculation methods were as follow: body trunk index = heart girth / body length × 100, body length index = body length / body height × 100, heart girth index = heart girth / body height × 100, cannon circumference index = cannon circumference / body height × 100, hip index = chest width / hip width × 100, chest width index = chest width / chest depth × 100 (Goren et al., 2006). Genomic DNA and RNA isolation, quality identification, and cDNA synthesis were the same as described in our previous studies (X. Zhang et al., 2015; Cui et al., 2018).

2.3. Identification of cattle PITX2 alternative splicing

In silico cloning and RT-PCR methods were used to identify novel spliced variants of the cattle *PITX2* gene. *In silico* cloning (also known as "electronic cloning" or "electronic hybrid") is a sequence analysis method based on network databases. This method uses computer technology and bioinformatics method to elongate a query sequence against a network database to predict the complete RNA sequence or alternative splicing products (Golshani et al., 2015). Specifically, we searched for the *PITX2* gene sequences of different species in the National Center for Biotechnology Information (NCBI) HomoloGene database and made multiple sequence alignments to identify a short, conserved mRNA sequence, which was then used as the query sequence for a BLAST search of highly similar ESTs in the EST database using NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Contig Express software was subsequently used to assemble the identified ESTs to form an EST contig. Finally, using this contig as a query, the prior steps were repeated until no contig was available to be extended. The last contig generated was thus the predicted full-length mRNA sequence. Different contigs may represent different spliced variants.

Based on the *in silico* cloning results and the cattle *PITX2* mRNA sequences found in the NCBI database, four pairs of primers (P1–P4) were designed to clone the entire coding region of *PITX2* (Accession number: NM_001097991.1) (P1) and to confirm whether the two predicted *PITX2* mRNA (XM_005207601.3; XM_005207602.3) were actually exist (P2–P4) (Fig. 1). The cDNA extracted from the nine different tissues of the three foetuses were pooled and used for transcript identification, using the same RT-PCR amplification system and program described in our previous study (S. Zhang et al., 2015a). The RT-PCR products were analysed *via* agarose gel electrophoresis and purified using a Gel Extraction Kit (Sangon Biotech, Shanghai, China), following which the products were combined with pMD19-T Vector (TaKaRa, Dalian, China) and transferred into competent cells DH5α (Tiangen Biochemical Technology Co., Ltd., Beijing, China). After incubating, target bands were sequenced using Gen-Script Co., Ltd. (Nanjing, China) (S. Zhang et al., 2015a).

2.4. Bioinformatic analyses of cattle PITX2 spliced variants

The nucleotide and protein sequences of transcripts were analysed using BioXM 2.6 (Nanjing Agricultural University, Nanjing, China) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast). The Smart program (http://smart.embl-heidelberg.de/) and SWISS-MODEL (https://www.