Gene 534 (2014) 356–361

Contents lists available at ScienceDirect

Gene

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

Intragenic DNA methylation status down-regulates bovine *IGF2* gene expression in different developmental stages

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ARTICLE INFO

Article history:
Accepted 26 September 2013
Available online 16 October 2013

Keywords:
Cattle
IGF2
DMR
DNA methylation
qPCR

ABSTRACT

DNA methylation is a key epigenetic modification in mammals and has an essential and important role in muscle development. Insulin-like growth factor 2 (*IGF2*) is a fetal growth and differentiation factor that plays an important role in muscle growth and in myoblast proliferation and differentiation. The aim of this study was to evaluate the expression of *IGF2* and the methylation pattern on the differentially methylated region (DMR) of the last exon of *IGF2* in six tissues with two different developmental stages. The DNA methylation pattern was compared using bisulfite sequencing polymerase chain reaction (BSP) and combined bisulfite restriction analysis (COBRA). The quantitative real-time PCR (qPCR) analysis indicated that *IGF2* has a broad tissue distribution and the adult bovine group showed significant lower mRNA expression levels than that in the fetal bovine group ($P<0.05$ or $P<0.01$). Moreover, the DNA methylation level analysis showed that the adult bovine group exhibited a significantly higher DNA methylation levels than that in the fetal bovine group ($P<0.05$ or $P<0.01$). These results indicate that *IGF2* expression levels were negatively associated with the methylation status of the *IGF2* DMR during the two developmental stages. Our results suggest that the methylation pattern in this DMR may be a useful parameter to investigate as a marker-assisted selection for muscle developmental in beef cattle breeding program and as a model for studies in other species.

1. Introduction

Epigenetics refers to the heritable changes that modify DNA or associated proteins without changing the DNA sequence itself (Egger et al., 2004). DNA methylation is a major epigenetic modification of the genome and is crucial for genomic stability. DNA methylation is a normal process used by mammalian cells in maintaining a development and has been implicated in diverse processes, including embryogenesis, genomic imprinting, X-inactivation and transposon silencing in mammals and plants (Lippman et al., 2004; Rhee et al., 2002). DNA methylation modification plays important roles in genome reprogramming and expression of genes that control animal development. Generally, methylation of CpG islands represses the initiation of transcription in the somatic cells of mammals and other vertebrates (Bird, 2002; Plass and Soloway, 2002). CpG sites are roughly 80% depleted in the genome, and are asymmetrically distributed into CpG poor regions and dense regions called CpG "islands" (CGI), which are often located in the promoter regions of roughly half of all the protein-coding genes (Takai and Jones, 2002). CGIs normally remain unmethylated, whereas the sporadic CpG sites in the rest of the genome are normally methylated (Chuang and Jones, 2007). Methylation of CGIs in promoter regions is often associated with gene silencing, and aberrant DNA methylation occurs in most cancers, leading to the silencing of some tumor suppressor genes (Jones and Baylin, 2002). Promoter methylation is negatively correlated with the gene expression level, indicating its suppressive role in regulating gene transcription (Li et al., 2011). DNA methylation is enriched in the gene body regions and the repetitive sequences, and depleted in the transcription start site (TSS) and the transcription termination site (TTS) (Li et al., 2011). The differentially methylated regions (DMRs) in promoters are highly associated with obesity development via expression repression of both known obesity-related genes and novel genes (Li et al., 2012).

Insulin-like growth factor 2 (*IGF2*) is a fetal growth and differentiation factor that plays an important role in muscle growth and

Abbreviations: 3'-UTRs, regulation at the 3' untranslated regions; AB, adult bovine; bp, base pair(s); ANOVA, analysis of variance; BSP, Bisulfite sequencing PCR; cDNA, Complementary DNA; COBRA, combined bisulfite restriction analysis; CpG, Cyt osineguan inedinucleotide; CTCF, CCCTC binding factor; DMR, differentially methylated region; FB, fetal bovine; H19, histocompatibility gene; IGF2, Insulin-like growth factor 2; LDM, longissimus dorsi muscle; miRNA, microRNA; qPCR, quantitative real-time PCR; QTLs, quantitative trait locus; QUMA, quantification tool for methylation analysis; SEM, standard error; SPSS, statistical product and service solutions; TSS, transcription start site; TTS, the transcription termination site.

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0378-1119/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.gene.2013.09.111

myoblast proliferation and differentiation (Giannoukakis et al., 1993). The *IGF2* gene contains 10 exons in all species studied to date. The mature form of *IGF2*, however, contains only the last three exons (exons 8, 9, and 10), with part of the translated product of exon 10. The other exons, together with four promoters (P1–P4), are involved in tissue- and/or developmental stage-specific expression of *IGF2* (Curchoe et al., 2005). In most bovine fetal tissues, only the paternal allele of this gene is expressed, while the maternal allele is transcriptionally silent (Dindot et al., 2004). Simultaneously, biallelic expression driven by promoters 3 and 4 has been observed in adult tissues (Curchoe et al., 2005).

The *IGF2* gene was the first imprinted genes to be identified in mammals. There were three DMRs that have been identified in the mouse *IGF2* gene: maternally methylated DMR0 located in exon U1, and paternally methylated DMR1 and DMR2 located in upstream of promoter 1 and exon 6, respectively (Feil et al., 1994; Moore et al., 1997). In humans, the regions homologous to mouse DMR0 and DMR2 have been shown to be differentially methylated, whereas the DMR1 is not methylated (Cui et al., 2002; Sullivan et al., 1999). The results of DMR1 and DMR2 knockout mice tests demonstrated that DMR1 has a silencer function (Constancia et al., 2000) and DMR2 has an activator function (Murrell et al., 2001). Regarding the control of *IGF2* expression, the model of *IGF2*/*H19* locus has the chromatin loop and has been proposed to explain the interactions among DMRs in this locus (Murrell et al., 2004). This DMR that contains CCCTC binding factor (CTCF) binding sites is located in the *IGF2 and histocompatibility* (*H19*) locus upstream of the H19 promoter, and CTCF-binding proteins regulate transcription of both genes. *IGF2* is expressed in the paternal chromosome with a methylated DMR and *H19* is expressed in the maternal chromosome with an unmethylated DMR (Chao and D'Amore, 2008; Killian et al., 2001; Zhang et al., 2004).

The bovine *IGF2* gene was localized to bovine chromosome 29, which harbors quantitative trait locus (QTLs) for meat, milk, and health traits in cattle (Casas et al., 2003; Schulman et al., 2004). It is a potent mitogen that is involved in placental and fetal development (Chao and D'amore, 2008; Curchoe et al., 2005). A different methylation pattern was identified within a region of the last exon of the bovine and porcine *IGF2* gene, suggesting the presence of an intragenic DMR, and it has been shown to be more highly methylated in spermatozoa than in oocytes (Gebert et al., 2006; Han et al., 2008). This methylated DMR is involved in the initiation of *IGF2* transcription, contributing to a high rate of gene transcription (Murrell et al., 2001).

This study is the first to compare the DNA methylation profiles in the DMR of the last exon of the *IGF2* gene in six tissues and organs (muscle, heart, liver, spleen, lung and kidney) and their relationships to mRNA expression patterns of fetal and adult using two-tail samples of Chinese Qincuan beef cattle breed with different growth performance. The objective was to identify the relation between epigenetic modifications and gene expression changes in cattle, which possibly contributed to animal breeding and genetics.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised 2004) and approved by the Institutional Animal Care and Use Committee (College of Animal Science and Technology, Northwest A&F University, China). Bovine embryos of slaughtered Qinchuan cows were collected from Tumen Abattoir, a local slaughterhouse of Xi'An, China. Adult Qinchuan cattle were obtained from Shannxi Kingbull Animal Husbandry Co. Ltd. (Baoji, China). Adult animals were allowed access to feed and water ad libitum under normal conditions and were humanely sacrificed as necessary to ameliorate suffering.

2.2. Tissue samples

Samples of six tissues and organs (longissimus dorsi muscle, heart, liver, spleen, lungs, and kidney) from 8 male individuals (4 individuals per stage) were harvested for RNA and DNA isolated within 10 min after slaughter at two key stages of myogenesis and muscle maturation: 90 days at embryo (fetal bovine, FB), and 24-month-old (adult bovine, AB). Fetal age (gestation period 280 days) was estimated based on crown-rump length (Richardson et al., 1990). There is no direct and collateral blood relationship within the last 3 generations among these cattle from each group. All fresh tissue samples were collected and divided into 1.5 mL plastic centrifuge tubes (each sample weighing around 100 mg) and immediately frozen in liquid nitrogen and stored at −80 °C until RNA and DNA extraction.

2.3. RNA preparation and quantitative real-time PCR (qPCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The integrity of the total RNA was checked by running 0.8% agarose gel and the concentration was determined by using a NanoDrop spectrophotometer (ND-1000, Wilmington, DE). Approximately 2 μg of total RNA was mixed with 2 μL of oligo $(dT)_{18}$ (0.25 μg/μL), 4 μL of dNTPs (2.5 mM each) and 5.4 μL of RNase-free ddH_2O and incubated at 65 °C for 5 min, then placed in ice bath for 2 min, and then 4 μL of 5× first-strand buffer, 1 μL of DTT (0.1 M), 1 μL of RNase inhibitor (40 U/μL) and 0.6 μL of superscript III reverse transcriptase (200 U/μL) (Invitrogen, Carlsbad, CA, USA) were added and incubated at 50 °C for 55 min and 70 °C for 15 min. The cDNA samples were stored at −20 °C.

The qPCR was performed using the SYBR Premix Ex Taq kit (Takara, Dalian, China) on a CFX96 Real-Time PCR detection system (Bio-Rad, USA). The primers used are given in Table 1. Reactions were carried out in a 25 μL volume containing 12.5 μL of 2× SYBR Green PCR Master Mix. The thermal profile for the qPCR was 95 °C for 3 min followed by 40 cycles of 95 °C for 20s, 60 °C for 30 s and 70 °C for 30 s. Data were normalized to the geometric mean of data from bovine *GAPDH* and

Table 1
Primers used for BSP and qPCR analyses.

Gene	Primer sequences (5′-3′)	Accession no.	Annealing (°C)	Product size (bp)
IGF2-DMR	F: TAATATGATATTTGGAAGTAGT R: ACATTTTTAAAAATATTATTCT	EU518675.1	50.4	420
IGF2-qPCR	F: TCTGTGCGGCGGGGAGCTGGT R: AGTCTCCAGCAGGGCCAGGTCG	EU518675.1	60.0	154
GAPDH-qPCR	F: CGACTTCAACAGCGACACTCAC R: CCCTGTTGCTGTAGCCAAATTC	NM_001034034	60.0	118
ACTB-qPCR	F: GTCATCACCATCGGCAATGAG R: AATGCCGCAGGATTCCATG	BT030480.1	60.0	84

F: forward primer; R: reverse primer.