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Association of a hypoxia-inducible factor-3α gene polymorphism with superovulation traits in Changbaishan black cattle

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ABSTRACT. This study was designed to examine a single nucleotide polymorphism (SNP) in the *HIF-3α* gene in three hundred Changbaishan black cattle using PCR-restriction fragment length polymorphism to determine whether there is an association between this SNP and superovulation. The cloning and sequencing results indicate that the polymorphism is due to a point mutation at the 278-bp position in the *HIF-3α* gene, resulting in 3 genotypes (AA, AB, and BB). Association analysis indicated that the polymorphism has a significant effect on the number of unfertilized embryos (NUE) ($P < 0.05$) in the cattle. Cattle with genotype BB had a higher NUE than those with genotype AA, but the difference in NUE between AB and AA or BB was not significant. The polymorphism

also has a highly significant effect on the number of degenerative embryos (NDE) and the number of total embryos (NTE) ($P < 0.01$). Genotype BB was associated with a higher NDE than AA, but the difference in NDE between AB and AA or BB was not significant. Genotype BB showed a higher NTE than AA or AB, but the difference in NTE between AA and AB was not significant. No significant conclusions could be drawn with respect to susceptibility to other traits. *HIF-3α* could serve as a useful biomarker for donor selection, superovulation improvement, and assisted fertility.

Key words: *HIF-3α*; Polymorphism; Superovulation traits; Cattle

INTRODUCTION

Superovulation is a major component of successful embryo transfer techniques that are used worldwide to produce valuable bovine embryos for breeding (Mapletoft and Hasler, 2005). However, the total number of transferable embryos has not changed markedly in the last 20 years, and the application of multiple ovulation and embryo transfer (MOET) technologies in animal industries is approaching a plateau. The major limitations to the development of MOET are the reliance on follicle stimulating hormone (FSH)-induced superovulation and the large variability in animal response to treatments (Hasler, 2003). Improvements in reproduction by traditional selective breeding methods have proved to be difficult due to low heritability and long reproductive cycles. It has been indicated that single nucleotide polymorphisms (SNPs) may become the preferred predictive marker of ovarian response (Fauser et al., 2008). The candidate gene approach provides an early breeding tool that could accelerate improvements in reproduction.

It is well known that oxygen homeostasis is fundamental for the maintenance of proper cellular functions and deregulation of oxygen dependent cellular processes is associated with developmental disorders. The central regulator of the mammalian oxygen-sensing pathway is a family of transcription factors called hypoxia-inducible factors (HIF), which trans-activate the expression of more than 60 genes in response to compromised oxygen tension (Maynard and Ohh, 2004, 2007).

HIF is a heterodimeric complex composed of an α or β subunit (Maynard and Ohh, 2004, 2007). To date, three α subunits (*HIF-1α*, *HIF-2α*, and *HIF-3α*) and one β subunit (*HIF-1β*, also called aryl hydrocarbon receptor nuclear translocator) have been identified (Wang et al., 1995; Wenger and Gassmann, 1997).

Hypoxia has been shown to have no effect on mRNA levels of *HIF-1α*, *HIF-1β*, and *HIF-2α*, but *HIF-3α* mRNA levels increased in all organs examined after 2 h of hypoxia (Heidbreder et al., 2003). Induction at the transcriptional level is a unique feature of *HIF-3α*, which may represent a rapidly reacting component of the HIF system for protection against hypoxic damage (Heidbreder et al., 2003).

Of the three hypoxia-inducible transcription factor family members identified in mammals so far, *HIF-3α* is the least characterized and understood. Interestingly, *HIF-3α* is most highly expressed in the human placenta and myocardium (Maynard et al., 2003). Because the amount of oxygen supplied from maternal blood to embryos through the placenta is limited, embryos are continuously exposed to lower oxygen tension (Genbacev et al., 2001). The availability of oxygen is limited in the uterus and embryos are continuously hypoxic (Lee et al., 2001). Under such conditions, it is likely that HIF-α proteins are not degraded but accumulate in the nucleus.

Given that both *HIF-1α* and *HIF-2α* are required for early embryonic development (Iyer et al., 1998; Tian et al., 1998; Peng et al., 2000), HIF activity must be elaborately regulated in embryos.

The *HIF-3α* transcript was expressed in virtually all embryonic and adult tissues examined. Gene expression analysis by RT-PCR revealed that *HIF-3α* mRNA is broadly expressed in various tissues of the mouse during embryonic and neonatal stages and that *HIF-3α* expression gradually decreases at 15 days postnatal (Yamashita et al., 2008).

Consequently, *HIF-3α* was chosen as a candidate gene for association with superovulation traits in the current study. Here, SNPs were examined using PCR-restriction fragment length polymorphism (PCR-RFLP) sequencing and RT-PCR. The aim of this study was to clarify the association between the *HIF-3α* gene polymorphism and superovulation traits in Changbaishan black cattle and to detect mRNA expression levels of *HIF-3α* at different early embryo developmental stages. These results validate the involvement of *HIF-3α* in superovulation responsiveness.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma (Shanghai, China) unless otherwise noted.

Superovulation procedure and embryo collection

Changbaishan black cattle are beef cattle that originated from an introduced Japanese black cattle line hybridized with local cattle. Our experiments were conducted from June to September in Jilin, China (127.33°E, 43.67°N). Before the superovulation treatment, Changbaishan black cattle, including nulliparous (ages 11 to 13 months) and multiparous cattle (ages 24 to 48 months), were selected by trans-rectal palpation for the absence of gynecological abnormalities. All the cattle received *ad libitum* feeding with a special high-protein, high-energy diet.

Superovulation was induced in the selected cattle using a protocol from the Beijing AnBo Embryo Biotech Center, the 16-day FSH - CIDR [EAZI Breed™ CIDR (progesterone), cattle insert] - prostaglandin (PG)- luteinizing hormone releasing hormone (LHRH) (FSH-CIDR-PG-LHRH) method, which is described in detail in the **Supplementary File**. Each cattle in estrus received two doses of frozen semen, which was collected by artificial vagina from one fertile Wagyu bull at 12-h intervals.

An experienced technician recovered the embryos and ova using a standard nonsurgical uterine flushing technique 6 days after artificial insemination. The initial search and evaluation of embryos and ova were carried out using a stereomicroscope at magnifications of 10X to 70X. Recovered viable embryos were evaluated at 70X magnification on the basis of the compactness and homogeneity of the cell mass, and cell assessment was conducted according to the published paper by Lindner and Wright (1983).

Briefly, cells were classified as M1 (morulae, Grade 1: embryos with single or small extruded blastomeres comprising less than 15% of the total cellular material), M2 (morulae, Grade 2: large cells or individual blastomeres extruded from the embryonic mass that make up more than 15% but less than 50% of the total cellular material), or blastocoel. Embryos with blastomeres that contained nuclei but were too underdeveloped to be considered viable were classified as degenerate. An unfertilized ovum was designated when there was no indication of cleavage or when all cytoplasmic fragments lacked nuclei.

Experiments were performed in accordance with the ‘Guiding principles in the use of

animals', adopted by the Chinese Association for Laboratory Animal Sciences. The study plan was approved by the Ethics Committee on the Use and Care of Animals, Jilin University.

Genomic DNA extraction

Approximately 2 mL blood per cattle was aseptically collected from the jugular vein immediately after recovery of the embryos, maintained in a tube containing ethylene diamine tetraacetic acid, and held on ice until delivery to the laboratory where it was stored at -20°C.

Genomic DNA was extracted using the Blood Genomic DNA Miniprep kit (Axygen, Hangzhou, China) and detected using 0.8% agarose gel electrophoresis. The DNA samples were dissolved in TE buffer and stored at -80°C for future use.

Primer synthesis and PCR amplification

Primers were designed based on the *Bos taurus* breed Hereford chromosome 18 sequence using the Oligo 6.0 software (http://www.oligo.net/). The upstream primer was 5'-CTGGGCAGTTG CTACTGTTCCTAT-3' and the downstream primer was 5'-AGTCCCGTCCAGGATTGGT-3'. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China.

The PCR mixture contained 50 ng DNA template, 10 pM upstream and downstream primer, 2.5 mM dNTP mixture, 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase in a 20-μL reaction volume. Amplification conditions consisted of 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 10 min. The amplification products were run on a 1% agarose gel and visualized using a UV transilluminator.

Genetic variation identification and sequencing

To detect the *HIF-3α* SNP, PCR products were digested with the restriction endonuclease *Alw44 I* (MBI Fermentas Life Science, Ontario, Canada) according to the manufacturer protocol. The mixture contained 5 μL PCR product, 10X digestion buffer, and 10 U enzyme, and was digested at 37°C for 8 h. Fragments were run on a 2% agarose gel and stained with ethidium bromide. Three genotypes were classified based on the band patterns. PCR products showing homozygous genotypes were cloned and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.

Statistical analysis

Associations between *HIF-3α* genotypes and superovulation traits were analyzed using the general linear model (GLM) procedure of SPSS version 13.0. The linear model is:

$$Y_{ijk} = \mu + P_i + M_j + G_k + E_{ijk} \quad \text{(Equation 1)}$$

where, Y_{ijk} is the observation for superovulation traits, μ is the overall population mean, P_i is the fixed effect due to the i-th parity, M_j is the fixed effect of j^{th} months of age, G_k is the fixed effect associated with k^{th} genotype (AA, AB and BB genotypes), and E_{ijk} is random error. The significance of differences was tested using Duncan's multiple comparison.

RESULTS

Genotypes

HIF-3α gene fragments were amplified from genomic DNA of all samples (Figure 1A). According to the three band patterns observed after the digestion reaction, the cattle were classified into three groups: AA, AB, and BB. The C278G transition results in an amino acid change from proline to arginine, a polymorphism that destroys the restriction site recognized by the *Alw44I* endonuclease. As shown in Figure 1B, DNA restriction fragments at locus C278G were generated by the *HIF-3α*-Alw44I polymorphisms: 379 bp for the AA genotype (40 cattle), 101 and 278 bp for the BB genotype (68 cattle), and 101, 278, and 379 bp for the AB genotype (192 cattle). The sequencing results are shown in Figures 2A and B-D.

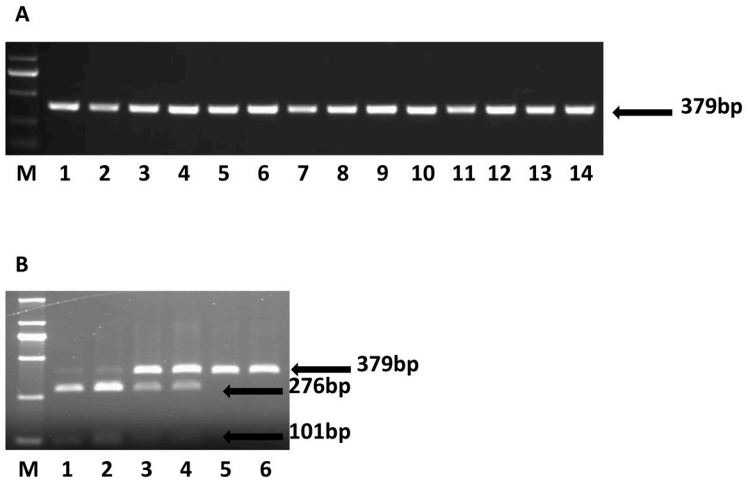

Figure 1. PCR-restriction fragment length polymorphism (RFLP) results for the HIF-3α gene in Changbaishan black cattle. **A.** PCR products of *HIF-3α* gene fragments analyzed using 1% agarose gel electrophoresis. *Lanes 1-14* refer to 14 PCR products selected from random samples. The arrow shows the expected 379-bp band of the *HIF-3α* gene fragments amplified from the genomic DNA of all samples. **B.** Representative RFLP genotypes of the *HIF-3α* gene fragment analysis using 2% agarose gel electrophoresis. *Lanes 5* and *6*, with one band at 379 bp, refer to the AA genotype, *lanes 1* and *2*, with two bands at 101 and 278 bp, refer to the BB genotype, *lanes 3* and *4*, with three bands at 101, 278, and 379 bp, refer to the AB genotype, *lane M* refers to the DL2000 marker.

Associations of genotypes with superovulation traits

Association analysis between *HIF-3α* genotypes and superovulation traits are shown in Table 1. The three genotypes showed no significant difference in the number of available embryos (NAE), the number of M2 embryos, and the number of M1 embryos. The BB genotype had a greater number of unfertilized embryos than the AA genotype (2.23 ± 0.316 versus 0.80 ± 0.197; $P < 0.05$). The BB genotype also had a greater number of degenerative embryos than the AA genotype (3.97 ± 0.491 versus 2.03 ± 0.461; $P < 0.01$). The AB genotype did not differ significantly from the AA or BB genotype for either trait. Additionally, the BB genotype had a greater number of total embryos (NTE) than either the AA or the AB genotype ($P < 0.01$ and $P < 0.01$, respectively).

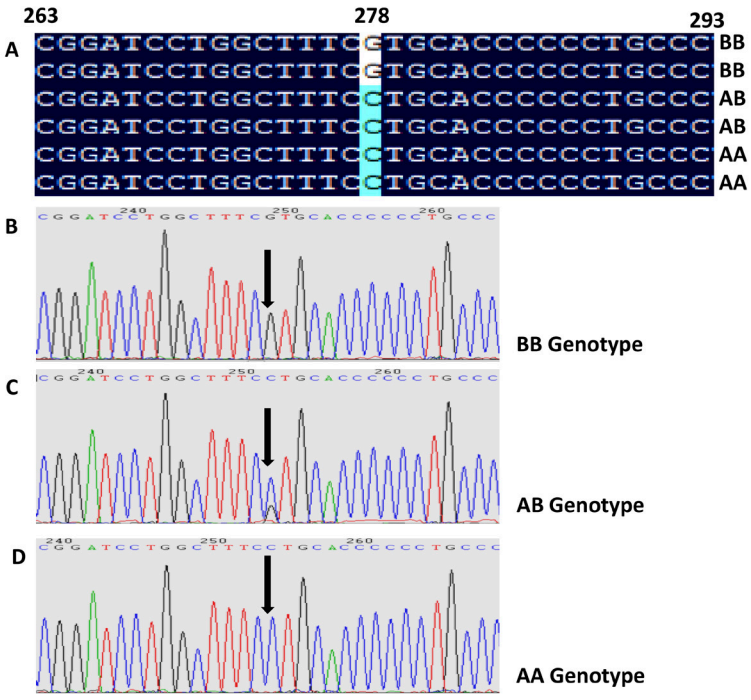

Figure 2. Sequencing results and sequence alignments of different genotypes in Changbaishan black cattle. **A.** Sequence alignment of the AA, AB, and BB genotypes. Sequence alignment shows a C278G transition in the *HIF-3α* gene fragments. **B.-D.** Sequencing of HIF-3α gene fragments. Representative sequencing results of the different genotypes: **B.** BB genotype, **C.** AB genotype, and **D.** AA genotype. The arrows indicate the mutation site and there are double peaks in the heterozygous AB genotype.

Table 1. Association of genotypes for *HIF-3α* with superovulation traits in Changbaishan Black cattle.

Superovulation traits	Genotype AA	Genotype AB	Genotype BB
NUE	0.80 ± 0.197^{a}	1.70 ± 0.198^{ab}	2.23 ± 0.316^{b}
NDE	2.03 ± 0.461^{A}	2.75 ± 0.212^{AB}	3.97 ± 0.491^{B}
NAE	7.95 ± 0.728	8.03 ± 0.488	10.12 ± 1.025
NTE	10.78 ± 1.036^{A}	12.44 ± 0.551^{A}	16.32 ± 1.225^{B}
NM2	1.10 ± 0.286	1.66 ± 0.162	1.79 ± 0.230
NM1	5.90 ± 0.572	6.06 ± 0.401	7.88 ± 0.895
NBE	0.95 ± 0.377^{a}	0.31 ± 0.077^{b}	0.45 ± 0.147^{a}
PUE	8.99% ± 2.920%	14.49% ± 1.494%	15.90% ± 2.592%
PDE	15.95% ± 2.737%	23.10% ± 1.542%	24.65% ± 2.722%
PAE	75.06% ± 4.187%^{a}	63.16% ± 2.319%^{b}	59.57% ± 3.489%^{b}
PM1	67.36% ± 4.466%^{a}	56.98% ± 2.430%^{ab}	51.89% ± 3.432%^{b}
PBE	6.55% ± 2.394%^{A}	1.97% ± 0.045%^{B}	3.50% ± 1.161%^{AB}

Superovulation traits correspond to the stereomicroscope observation. NUE = number of unfertilized embryos; NDE = number of degenerated embryos; NAE = number of available embryos; NTE = number of total embryos; NM2 = number of M2 embryos; NM1 = number of M1 embryos; NBE = number of blastulas; PUE = percentage of unfertilized embryos = NUE/NTE x 100%; PDE = percentage of degenerated embryos = NDE/NTE x 100%; PAE = percentage of available embryos = (NM1+NM2+NBE)/NTE x 100%; PM1 = percentage of M1 embryos = (NM1+NBE)/NTE x 100%; PBE = percentage of blastulas = NBE/NTE x 100%. Values with different lowercase letter superscripts in the same column differ significantly ($P < 0.05$). Values with different uppercase letter superscripts in the same column differ significantly ($P < 0.01$).

DISCUSSION

Gene sequences and variations among them are the entry points for understanding gene expression and function (Liu et al., 2009). The function of transcriptional regulation may be changed by point mutations in the regulatory regions of genes that can alter configurations (Mayo et al., 2006). Using genetic screening, mutations involving defects in transcriptional regulation have been found for developmentally important genes (Gibson and Honeycutt, 2002). Mutations can be a useful tool for animal breeding and production due to such alternations in transcriptional regulation. However, few mutations have been discovered compared to the number of genes.

Maintaining oxygen homeostasis is essential to all higher organisms for proper development, growth, and maintenance of tissue structural integrity. In general, HIFs are vital to proper development and deletion of the *HIF-1α* gene results in prenatal death. HIFs are crucial for oxygen homeostasis during both embryonic development and postnatal life (Yamashita et al., 2008).

The third α-class of HIF subunits was first isolated in the mouse (mHIF-3α) in 1998 (Gu et al., 1998). The human homologue of the *mHIF-3α* (*hHIF-3α*) was later isolated and shown to have high homology to *hHIF-1α* and *-2α* (Hara et al., 2001), raising the possibility that it may also be involved in adaptive responses to changes in oxygen tension. The protein encoded by this gene is the alpha-3 subunit of one of several alpha/beta-subunit heterodimeric transcription factors that regulate many adaptive responses to low oxygen tension. The alpha-3 subunit lacks the trans-activation domain found in factors containing either the alpha-1 or alpha-2 subunits. It is thought that factors containing the alpha-3 subunit are negative regulators of hypoxia-inducible gene expression. *HIF-3α* is also a specific target of *HIF-1α*, but is not affected by *HIF-2α* knockdown (Hara et al., 2001). Our experiments were designed to locate and identify SNPs in *HIF-1α* exons, but we found no SNP sites in our cattle herds. We hypothesize that the SNP may have an effect on the binding of *HIF-3α* to *HIF-1α*, or that the SNP site is not in the binding zone. In contrast with *HIF-1α* and *HIF-2α*, *HIF-3α* is not regulated at the protein level. The HIF-3α protein can be detected under normoxic conditions in the cytoplasm and nuclei, but increases under hypoxic conditions (Tanaka et al., 2009). At least six different splice variants, *hHIF-3α* 1-6 (Maynard et al., 2003) may be expressed from the human *HIF-3α* locus, suggesting it may exert a primarily negative regulatory effect on hypoxic gene induction (Tanaka et al., 2009).

The functional properties of *HIF-3α* are still unknown. A study by Heidbreder et al. (2003) suggested that *HIF-3α* contributes to protection during early periods of hypoxia. *HIF-3α* was up-regulated by severe and/or sustained hypoxia and was strongly induced by hypoxia (1% O_2) both at the protein and mRNA level due to an increase in protein stability and transcriptional activation (Heidbreder et al., 2003). The hypoxic induction of *HIF-3α* protein is also dependent on the duration of the hypoxic conditions. *HIF-3α* protein increased rapidly within 30 min of exposure to 1% O_2 and increased further with a longer exposure to hypoxia, indicating that hypoxia stabilized this protein (Li et al., 2006). Low oxygen tension leads to the stabilization of the α-subunit, nuclear translocation, and recruitment of transcriptional co-activators (Kallio et al., 1998). In a further investigation, we attempted to locate the regulator of *HIF-3α* mRNA expression. As it is shown in **Figure S1**, we failed to detect expression of *HIF-3α* in the germinal vesicle oocyte or 16-cell embryo, and the 4-cell embryo had the highest expression, approximately 5.6-fold more than the metaphase II (MII) oocyte ($P < 0.01$). Among the five developmental stages, morulae had the lowest expression levels of *HIF-3α*, approximately 0.08-fold compared to MII oocyte levels ($P < 0.05$). MII oocytes, 2-cell, and 8-cell embryos had similar expression levels, indicating that there was little or no *HIF-3α* mRNA expression during these developmental stages. During folliculogenesis, artificial insemination

and early embryonic development, the oocyte/zygote is under normal oxygen tension. However, absence of *HIF-3α* mRNA expression during the 16-cell stage needs to be investigated further. During the 16-cell stage, the embryo crosses the 8 to 16 cell bovine embryonic blockade and is then able to transport oxygen to the inner blastomeres for normal metabolism; therefore, *HIF-3α* mRNA is down-regulated. The specific mechanism behind this process needs further investigation.

The 4-cell embryo had the highest expression of *HIF-3α* mRNA and the other three types of embryo had almost the same expression levels as each other. This is the first time that *HIF-3α* mRNA expression patterns in bovine embryos/oocytes have been reported. The low expression of *HIF-3α* mRNA in the morulae stage corresponds to its inferred function to prevent cells from differentiating. When the embryo reaches the blastocyst stage, it starts to differentiate into several mesoderms. However, when the embryo starts to differentiate, *HIF-3α* mRNA is down-regulated by regulatory mechanisms.

We conclude that oxygen tension was lowest during the 4-cell stage and highest during the 16-cell stage of development. The low oxygen tension during the 4-cell stage may be due to the long culture time. Lower expression of *HIF-3α* mRNA in the 8-cell stage compared to the 4-cell stage may be due to developmental heterogeneity because some of the embryos had passed, or were in the process of passing the blockade. Genetic and environmental factors may have also been involved. For hypoxic stabilization, the activity of *HIF-1α* is modulated by phosphorylation via the mitogen-activated protein kinases or the phosphatidylinositol 3-kinase pathway (Richard et al., 1999; Zhong et al., 2000).

In this study, we compared the association of a polymorphism with superovulation and found that cattle with genotypes AA and BB differ significantly in their superovulation traits. This is the first report to detect the association of *HIF-3α* mRNA expression with superovulation traits. The polymorphism of the *HIF-3α* gene has a significant effect on a number of superovulation traits. The numbers of cattle with AA, AB, and BB genotypes were 40, 192, and 68, respectively. It appears that the heterozygous genotype is more abundant and accounts for approximately two-thirds of all cattle in our herds. The genotype frequency of AA:AB:BB was approximately 1:4.8:1.7, and the allele gene frequency of A:B was approximately 1:1.2, indicating that AB is the dominant genotype and the B allele is the dominant allele. As shown in Table 1, all of the superovulation traits showed that the BB genotype cattle were the most abundant and AA genotypes were the least abundant. Based on NAE and NTE, the BB genotype may be better suited for daily production requirements.

We conclude that the *HIF-3α* gene may be involved in the whole process of superovulation treatment, especially during early embryonic development. *HIF-3α* influences the number of embryos as well as the corresponding percentage. *HIF-3α* may serve as a molecular marker in donor selection for superovulation treatment.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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