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Polymorphic CA microsatellites in the third exon of the bovine *BMP4* gene

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ABSTRACT. We examined the variation of the *BMP4* gene in four Chinese indigenous cattle breeds and investigated the association of this polymorphism with body measurement traits. Using PCR-SSCP and DNA sequencing, a polymorphic microsatellite was detected in the third exon of the bovine *BMP4* gene in 459 samples from four Chinese indigenous cattle breeds, Qinchuan, Luxi, Nanyang, and Jiaxian red. The two alleles were named A and B. Allele frequencies of *BMP4*-A/B in the four breeds were 0.939/0.061, 0.928/0.072, 0.929/0.071, and 0.938/0.062, respectively. Least squares analysis revealed significant effects of genotype on withers height in the four breeds, on hip height in two breeds (Luxi and Nanyang, $P < 0.05$) and on chest circumference in Qinchuan ($P < 0.05$), while no significant effects of genotype on body length and rump length were found. These results can be applied to marker-assisted selection of Chinese cattle breeds, but a much larger number of animals will be needed for association analysis.

Key words: *BMP4* gene; Body measurement traits; Cattle; Microsatellite

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta (TGF-β) superfamily (Wozney et al., 1988), which are multifunctional cytokines and are expressed in a variety of cells (Massague, 1998). In this superfamily, BMPs were originally identified on the basis of their ability to induce ectopic bone formation when implanted within soft tissue *in vivo* (Urist, 1965). So far, more than 20 members have been identified in the BMP family. They are similar in structure, but vary in different periods and different tissues. As one of the most important members of BMPs, *BMP4* is a multifunctional growth factor that is known to play an important role in skeletal development and bone formation (Hogan, 1996; Bellusci et al., 1996). It also plays a critical role during embryonic development, ranging from the establishment of the basic body plan to morphogenesis of individual organs (Miyazaki et al., 2000).

In adult life, *BMP4* is increased in concentration during fracture healing (Nakase et al., 1994; Shafritz and Kaplan, 1998). Polymorphism analysis has shown its association with bone density in humans (Mangino et al., 1999; Ramesh et al., 2005) and with growth traits in goats (Fang et al., 2009). These findings suggest a possible role of this protein in determining bone mass and structure, and possibly bone growth. However, there has not been much research on the polymorphism in bovine *BMP4* gene. Therefore, the objective of this study was to evaluate the variation of the *BMP4* gene in four Chinese indigenous cattle breeds and to investigate the association of this polymorphism with body measurement traits.

MATERIAL AND METHODS

Animals and DNA isolation

Four hundred and fifty-nine animals were used in this study. The animals originated from different farms: the Qinchuan cattle (QC, N = 197) were from the reserve farm of QC (Weinan city, Shaanxi Province), the Luxi cattle (LX, N = 104) were from the reserve center of LX (Heze city, Shandong province), the Nanyang cattle (NY, N = 85) were from the breeding center of NY (Nanyang city, Henan Province), and the Jiaxian red cattle (JXR, N = 73) were from the reserve farm of JXR (Jiaxian county, Henan Province). The following traits were measured as previously described (Gilbert et al., 1993), including body length, withers height, hip height, chest circumference, and rump length. In order to minimize systematic error, a single person was assigned to measure one of the five traits in all animals.

Blood samples from 459 individuals of four Chinese indigenous cattle breeds were obtained, treated with 2% heparin and stored at -80°C. DNA samples were extracted from blood samples according to standard procedures (Sambrook and Russell, 2002).

Primers and polymerase chain reaction conditions

Based on the bovine *BMP4* gene (GenBank accession No. NC 007308.3), one pair of polymerase chain reaction (PCR) primers (forward: 5' GAG TAT GAC AAG GTG GTT CTG 3' and reverse: 5' AAC ATT TGC ACG TAA AGT CA 3') was designed to amplify a 259-bp PCR product in exon 3.

Polymerase chain reaction amplifications were performed in a 20-μL reaction mixture containing 50 ng DNA template, 10 pM of each primer, 0.20 mM dNTP, 2.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (TaKaRa, Dalian, China). The cycling protocol was 5 min at 95°C, 32 cycles of 94°C for 30 s, 58.3°C annealing for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. PCR products were electrophoresed on 1.5% agarose gels (containing 200 ng/mL ethidium bromide) using 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA).

Single-stranded conformation polymorphism

PCR products were analyzed for single-strand conformation polymorphisms (SSCP). Aliquots of 6 μL of the above PCR products were mixed with 6 μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), incubated at 98°C for 10 min and then chilled on ice. Denatured DNA was loaded onto 14% PAGE gel in 1X TBE buffer and constant voltage 110 V for 12 h. The gel was stained with 0.1% silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde) according to Zhang et al. (2007).

Cloning and sequencing

Based on the patterns of SSCP, PCR products with each different pattern were cloned: the assumed polymorphic DNA samples (3 of each type) were amplified and purified using a cleaning kit (Axy Prep™ DNA Gel Extraction Kit, Axygene), and the products were inserted into T4 vector (pGEM.T Easy) and then transferred to *Escherichia coli* DH5a strain. Positive clones were cultured and sequenced in ABI PRIZM 3730 DNA sequencer (Perkin-Elmer Shanghai Sangon Biological Engineering Technology, Ltd.). The DNAMAN software (version 6.0) was used to analyze the sequences.

Statistical analysis

In these four Chinese indigenous cattle breeds, genotypic frequencies and allelic frequencies of the *BMP4* locus were calculated directly, and Hardy-Weinberg equilibrium and differences in genotypic frequencies were analyzed by the χ^2 test, which were performed using the SPSS software (version 17.0). Population genetic indices: gene heterozygosity, gene homozygosity, effective allele numbers, and polymorphism information content (PIC) were calculated according to Nei and Roychoudhury (1974) and Nei and Li (1979).

The SPSS software (version 17.0) was used to analyze the relationship between the genotypes and body measurement traits. The following linear model was used in the analysis:

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

where Y_{ijk} is the observation for the body measurement trait, μ is the overall population mean, A_i is the fixed effect of the ith age, G_j is the fixed effect of jth genotype (AA and AB genotypes), S_k is the fixed effect of sex, and E_{ijk} is the random error.

RESULTS

Genetic polymorphism of the bovine *BMP4* gene and the χ^2 *t*est

Using the PCR-SSCP method, expected product from the third exon of the *BMP4* gene exhibited two different patterns (Figure 1). We named them AA with two bands and AB with three bands. Table 1 shows that allelic frequencies in QC, LX, NY, and JXR varied from 0.061 to 0.939. The χ^2 test showed that genotype distributions in all four breeds detected agreed with Hardy-Weinberg equilibrium ($P > 0.05$) (Table 1), and genotypic frequencies in all four breeds were not different ($P > 0.05$). AA was the domain genotype in all populations studied (ranging from 0.856 to 0.878), while BB was not detected in all populations tested. Effective allele numbers and PIC were 1.129, 1.155, 1.151, 1.131 and 0.108, 0.125, 0.123, 0.110 in QC, LX, NY, and JXR, respectively (Table 1). According to the classification of PIC (low polymorphism if PIC value <0.25, medium polymorphism if 0.25 < PIC value < 0.5, and high polymorphism if PIC value >0.5), all four cattle breeds examined showed a low polymorphic level.

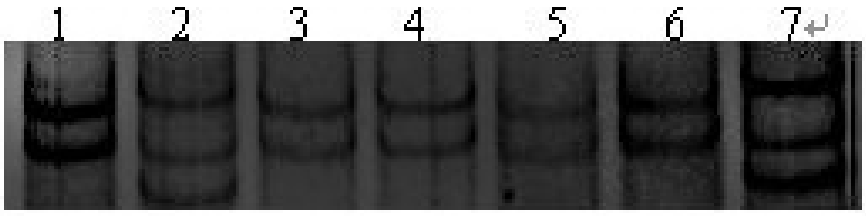

Figure 1. Electrophoresis patterns of PCR-SSCP exon 3 of the bovine *BMP4* gene. *Lanes 1*, *3*, *4*, *5*, and *6* = AA genotype; *lanes 2* and *7* = AB genotype.

Table 1. Genotypic and allelic frequencies and population genetic indices at the bovine *BMP4* gene locus in four breeds.

Breed	Genotypic frequencies (N)		Total	Allelic frequencies		Effective allele number	PIC	χ^2 (HWE)
	AA	AB		A	B			
QC	0.878 (173)	0.122 (24)	197	0.939	0.061	1.129	0.108	0.114
LX	0.856 (89)	0.144 (15)	104	0.928	0.072	1.155	0.125	0.027
NY	0.859 (73)	0.141 (12)	85	0.929	0.071	1.151	0.123	0.025
JXR	0.877 (64)	0.123 (9)	73	0.938	0.062	1.131	0.110	0.180

QC = Qinchuan cattle; LX = Luxi cattle; NY = Nanyang cattle; JX = Jiaxian red cattle; N = Number of observations. PIC = polymorphism information content; χ^2 (HWE) = Hardy-Weinberg equilibrium by the χ^2 test. Its P value was above 0.05.

Clonal sequencing analysis suggested the existence of a short sequence with more than 18 CA dinucleotide repeats, which was located at position 3470-3510 bp (the start nucleotide of exon 1 as the +1; GenBank accession No. NC007308.3), starting at 230 bp downstream from the termination site of the gene. Individuals with the AA genotype showed a CA dinucleotide repeat number of 19, and individuals with the AB genotype showed a CA dinucleotide repeat number of 19 and 18 (Figure 2).

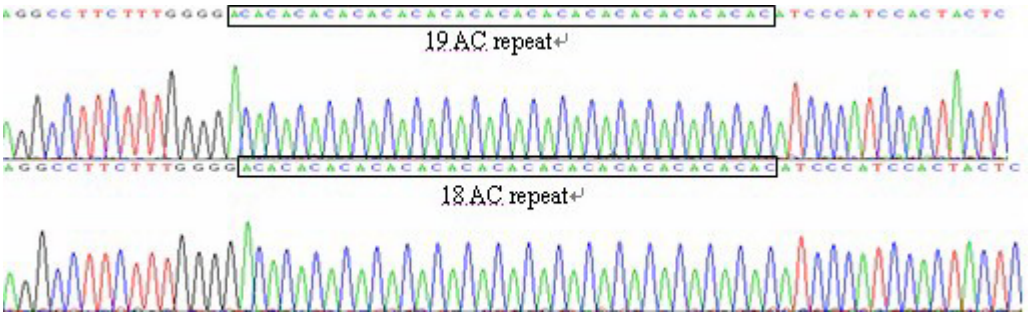

Figure 2. Sequencing map of the microsatellite for exon 3 in the bovine *BMP4* gene.

Effect of the polymorphism locus on body measurement traits

Five body measurement traits were analyzed by comparison between genotypes of 459 individuals and their phenotypic data. The results of association analysis of the gene-specific marker are shown in Table 2. There were significant effects on withers height in three breeds (LX, NY, JXR; $P < 0.05$), on hip height in two breeds (LX, NY; $P < 0.05$) and on chest circumference in QC ($P < 0.05$).

Table 2. Least square means and standard errors of the body measurement traits obtained for the genotypes of the *BMP4* polymorphism in Chinese indigenous cattle.

Breed	Genotype	Body measurement traits (cm)				
		BL	CC	HH	RL	WH
QC	AA	135.7 ± 2.2	174.2 ± 2.9[a]	126.2 ± 1.8	45.9 ± 0.9	121.7 ± 1.3[a]
	AB	134.4 ± 3.0	167.8 ± 3.8[b]	124.3 ± 2.4	44.4 ± 1.2	118.0 ± 1.7[b]
	P	0.585	0.032	0.303	0.104	0.006
LX	AA	142.6 ± 1.9	187.5 ± 2.3	131.7 ± 1.5[a]	46.2 ± 0.9	129.6 ± 1.4[a]
	AB	140.6 ± 3.0	184.4 ± 3.5	127.6 ± 2.3[b]	46.6 ± 1.4	125.8 ± 2.1[b]
	P	0.322	0.209	0.014	0.670	0.014
NY	AA	150.5 ± 0.6	184.0 ± 1.0	130.0 ± 0.5[a]	40.2 ± 0.3	131.6 ± 0.5[a]
	AB	149.8 ± 1.4	182.7 ± 2.7	127.5 ± 1.2[b]	38.6 ± 0.9	128.6 ± 1.3[b]
	P	0.671	0.643	0.032	0.079	0.025
JXR	AA	140.9 ± 3.0	173.8 ± 3.0	125.3 ± 2.4	42.3 ± 1.5	127.9 ± 2.0[a]
	AB	134.6 ± 3.9	168.1 ± 4.0	121.1 ± 3.1	42.1 ± 1.9	122.9 ± 2.6[b]
	P	0.051	0.082	0.096	0.900	0.022

Data are reported as means ± SEM. [a,b]Means with different superscript letters were significantly different ($P < 0.05$). BL = body length; CC = chest circumference; HH = hip height; RL = rump length; WH = withers height. For other abbreviations, see legend to Table 1.

DISCUSSION

The TGF-β signaling pathway is a fundamental and diversified ancient metazoan signal transduction engine, pivotal for animal tissue development and homeostasis. It has been reported that this gene is highly evolutionary conserved (Winnier et al., 1995). This study detected mutation in all three exons of the bovine *BMP4* gene. However, only one polymorphism was found in exon 3, which was not in the coding region. This implied that *BMP4*

signals may be essential for different growth and development processes. It was reported that congenital anomalies of the kidney and urinary tract arise in approximately half of mice with heterozygous mutation in *BMP4*, while homozygous null mutation in *BMP4* is lethal with mice dying *in utero*. Deficient *BMP4* mice also die at early embryonic stages, showing little or no mesodermal differentiation due to defects in mesodermal formation (Winnier et al., 1995).

In the TGF-β superfamily, growth differentiation factor 5 and *BMP4* were reported to be associated with body measurement traits in goats and cattle, respectively (Fang et al., 2009; Liu et al., 2010). In the present study, individuals with the AA genotype compared to individuals with the AB genotype showed significantly greater hip height, withers height and chest circumference ($P < 0.05$). The results agree in part with a previous study.

Homozygous null BB in *BMP4* was not detected in all populations tested, as the frequency of the B allele was very low, which is likely due to the limited sample size or maybe homozygous null BB is lethal. It has been proposed that GT, CA, CT, GA, GC, or AT repeat-binding proteins could participate in recombination processes by inducing Z-conformation or other alternative secondary DNA structure (Karlin et al., 1998; Biet et al., 1999). Microsatellite and mini-satellite DNAs have been proposed as hotspots for recombination (Jeffreys et al., 1998; Templeton et al., 2000). In humans (GenBank accession No. NM_001202.3), mice (GenBank accession No. NM_007554.2), horse (GenBank accession No. XM_001494966.2), sheep (GenBank accession No. EE85_1370), and goat (Fang et al., 2009), without exception, there is a microsatellite of dinucleotide-repeated (CA) sequence in the 3' flanking region of the *BMP4* gene, and maybe this can imply the functional significance of the microsatellite. The microsatellite may also be in linkage disequilibrium with variation in other regions of the gene with functional or structural significance (Karlin et al., 1998). As this microsatellite is located in the 3' flanking region of exon 3, it may also have the ability to affect the structure of mRNA and translation efficiency (De Smit and van Duin, 1994). We did not detect animals with 20 continuous CA dinucleotide repeats (as published in GenBank, accession No. NC_007308.3) in this locus. Maybe the locus is population specious, only existing in some indigenous breeds.

In summary, the most likely reason is that the mutation only exists in some indigenous breeds, and maybe this mutation has some crucial meaning for the breeds tested. Maybe *BMP4* is an important candidate gene that affects body measurement traits, but further investigations are essential for marker-assisted selection.

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