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A novel polymorphism of the *lactoferrin* gene and its association with milk composition and body traits in dairy goats

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ABSTRACT. Milk composition and body measurement traits, influenced by genes and environmental factors, play important roles in value assessments of efficiency and productivity in dairy goats. Lactoferrin (LF), involved in the efficient expression of protein in milk, is also an anabolic factor in skeletal tissue and a potent osteoblast survival factor. Therefore, it is an important candidate gene for milk composition and body measurement trait selection in marker-assisted selection. We employed PCR-SSCP and DNA sequencing to screen the genetic variations of the *LF* gene in 549 Chinese dairy goats. A novel single-nucleotide polymorphism (SNP) (G198A in exon II) of the *LF* gene was detected. The frequencies of the AA genotype were 0.0285 and 0.0261 in GZ and SN populations, respectively. Both populations were found to have low levels of polymorphism and were in Hardy-Weinberg disequilibrium ($P < 0.05$). We found significant ($P < 0.05$) associations

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of the SNP marker with milk protein and acidity in the total population; animals with the AA genotype had higher mean values for milk protein than those with the GA genotype. Animals with genotype AA had higher mean values for withers height than those with genotype GG ($P < 0.05$). We concluded that this SNP of the *LF* gene has potential as a genetic marker for milk composition and body traits in dairy goat breeding.

Key words: Goat; *LF* gene; SNP; PCR-SSCP; Milk composition; Body measurement traits

INTRODUCTION

Lactoferrin (LF) is an iron-binding glycoprotein that is closely related in structure to the iron-transport protein transferrin (Kinsella and Whitehead, 1989). The *LF* gene, together with transferrin and melanoma antigen p97, belongs to the *transferrin* gene family (Williams, 1982; Park et al., 1985). The three genes present strong homology between the pairs of exons and the splicing pattern, which firmly support the hypothesis that the *transferrin* gene family originated from a duplication of a common ancestral gene (Masson et al., 1966). *LF* is mainly present in external secretions such as milk; its expression is both ubiquitous and species, tissue, and cell-type specific (Cohen et al., 1987; Teng et al., 1987, 1989).

LF has been demonstrated to possess a multitude of biological functions. The study of *LF* structure shows that there are binding sites for Fe^{3+} in its two clefts and it is best known for its ability to bind iron (Bullen, 1972). *LF* has been recognized as an anti-infective agent, and over the past few decades a broad spectrum of antibacterial properties have been observed (Yamauchi et al., 1993; Jenssen and Hancock, 2009; Leon-Sicairos et al., 2009). Furthermore, bovine or human *LF* is an anabolic factor in skeletal tissue and a potent osteoblast survival factor (Cornish, 2004). It stimulates the proliferation of bone-forming cells, osteoblasts, and cartilage cells at physiological concentrations *in vitro*. Local injections of *LF* in adult mice resulted in increased calvarial bone growth, which further suggests that *LF* has a physiological role in bone growth and a potential therapeutic role in osteoporosis (Cornish, 2004; Cornish et al., 2005; Brandl et al., 2010). Furthermore, high-level expression of the human *LF* gene in milk of transgenic mice using genomic *LF* sequence demonstrates that the genomic *LF* sequence represents a valuable element for efficient expression of milk protein in transgenic animals (Kim et al., 1999). *LF* is also an immune system component (Yamauchi et al., 2006; Livney, 2010). A recent study found that bovine *LF* activated the transcription of important immune-related genes in the small intestine and such transcriptional activation might promote systemic host immunity (Yamauchi et al., 2006). Other functions of *LF* such as promoting DNA synthesis (Nichols et al., 1987), inhibiting tumor growth (Jeremy, 1995) and inhibiting liquid preoxidation (Gutteridge et al., 1981) have also been reported.

To our knowledge, few polymorphisms of the goat *LF* gene have been reported. Based on the multiple regulatory roles of the *LF* gene in organism, the *LF* gene is a potential gene for milk composition and body measurement traits in dairy goats. Therefore, the objective of this study is to detect SNPs of the *LF* gene in dairy goats and explore their possible association with milk composition and body measurement traits in two dairy goat breeds.

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MATERIAL AND METHODS

Sample collection and DNA extraction

A total of 549 adult dairy goats including the Guanzhong breed (GZ, N = 281) and Xinong Saanen breed (SN, N = 268) from Shaanxi province, China, aged three, were randomly selected from breeding populations. Milk samples from each goat were collected and immediately frozen at -20°C. Milk compositions including milk fat, protein, total solids (TS), solid-non-fat (SNF), lactose, and acidity were analyzed using an infrared milk analyser (Milko Scan 133B Analyser; Foss Electric, Hillerød, Denmark). Subsequently, 338 (GZ, N = 138 and SN, N = 200) of 549 animals were selected randomly for collection of body measurement traits, including withers height (WH), body length (BL), and chest circumference (CC). In order to minimize systematic error, the same person was assigned to measure one of the three traits in all animals. Genomic DNA was extracted from leukocytes using standard phenol-chloroform protocol.

PCR amplification

According to the sequence of the goat *LF* gene (GeneBank accession No. NC_FJ609300), one pair of polymerase chain reaction (PCR) primers was designed with primer 5.0 as follows: F: 5'-TGTAGAAGCCCTCACTCTTTG-3', R: 5'-CCTTCCCTTTCAGTCTGGTC-3'. This primer pair was used to amplify a 247-bp product of the *LF* gene exon II and its flanking region. PCR was performed on a 12.5-μL reaction mixture containing 25 ng genomic DNA, 0.25 μM of each primer, 1X buffer (including 1.5 mM $MgCl_2$), 100 μM dNTPs and 0.3125 U *Taq* DNA polymerase (MBI). The cycling protocol was 5 min at 95°C, 32 cycles of denaturing at 94°C for 30 s, annealing at 52.7°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 10 min.

Single-stranded conformation polymorphism (SSCP) and sequencing

Aliquots of 5 μL of the PCR product were mixed with 5 μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled on ice. Denatured DNA was subjected to polyacrylamide gel electrophoresis (80 × 73 × 0.75 mm) in 1X Tris-borate EDTA (TBE) buffer at constant voltage (200 V) for 2.5-3.0 h. The gel was stained with 0.1% silver nitrate (Lan et al., 2007). After the polymorphism was detected, the PCR products of different electrophoresis patterns were sequenced in both directions in an ABI PRIZM 377 DNA sequencer and the sequences were analyzed with the SeqMan software.

Statistical analysis

Based on the genotype number of the *LF* exon II and its flanking region in 549 goats analyzed, the following items were statistically analyzed according to the previous approaches (Nei and Roychoudhury, 1974; Nei and Li, 1979), including genotypic frequencies, allelic frequencies, Hardy-Weinberg equilibrium, gene homozygosity, gene heterozygosity, effective allele number, and polymorphism information content (PIC). The association between single-nucleotide polymorphism (SNP) marker genotypes of the *LF*

gene and records of body measurement traits (WH, BL and CC) and milk composition (fat, protein, TS, SNF, lactose, density, and pH value) was analyzed by the least-squares method as applied in the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA) according to the following statistical linear model:

$$Yijkl = \mu + Gi + BFk + \varepsilon ijkl$$

where Yijkl is the observation for the milk composition and body measurement traits, μ is the overall mean for each trait, Gi is the genotype effect, BFk is the fixed effects of breed and farm, and εijkl is the random environment effect.

RESULTS

After 247-bp products of the *LF* gene were amplified, polymorphism of the *LF* gene in 549 goats was detected by SSCP and confirmed by the DNA sequencing method. Three unique SSCP patterns for AA, AG and GG were detected in two dairy goat breeds (Figure 1). In order to better understand the detailed genetic variations of the *LF* gene, the three polymorphic SSCP patterns were sequenced (Figure 2). Comparison between the nucleotide sequences of the goat *LF* gene (GeneBank Accession No. NC_FJ609300) and the sequencing result revealed one novel SNP, named G198A. The G>A mutation, a synonymous mutation of arginine, is located at position 198 of the *LF* gene.

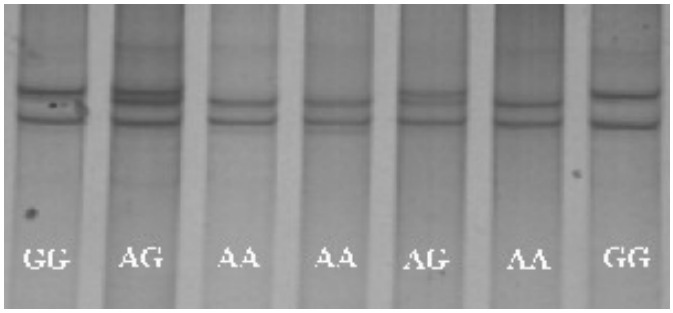

Figure 1. PCR-SSCP patterns of exon 2 locus of the *lactoferrin* gene.

With the sequence data from different individuals, three genotypes were described as GG, AA, and GA. The three genotypes corresponded to three polymorphic patterns found in this study. Moreover, allele frequencies of the SNP were investigated and performed by the χ^2 test in the two dairy goat breeds in our study (Table 1). The data demonstrated that the frequencies of G/A alleles were 0.9448/0.0552 and 0.9552/0.0448 in the GZ and SN breeds, respectively. The total frequencies of alleles G and A were 0.9499 and 0.0501. The frequencies of the AA genotype were 0.0285 and 0.0261 in GZ and SN populations, respectively, indicating that the frequency of the AA genotype was very low in the two breeds. This observation was made possibly by the occurrence of random genetic drift due to the low frequency of allele A. There was no significant difference in allelic frequencies and the two populations were in Hardy-Weinberg disequilibrium ($P < 0.05$).

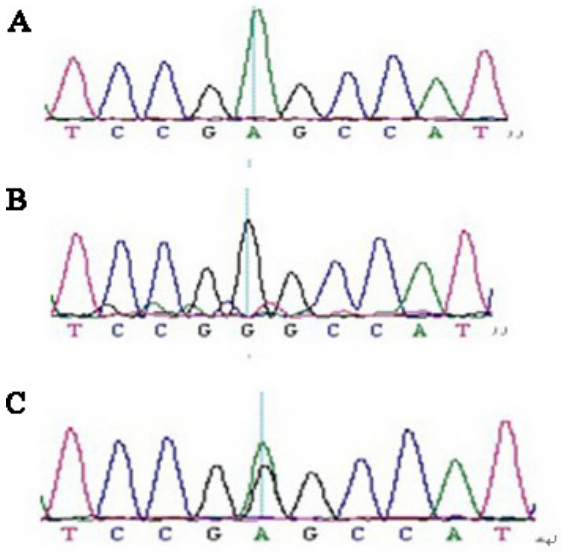

Figure 2. Sequencing maps of the goat *lactoferrin* gene from different genotypes. **A.** Genotype AA. **B.** Genotype GG. **C.** Genotype AG.

Table 1. Genotype frequencies (%) of the *LF* gene for the SNP in two goat populations.

Breeds	Observed genotypes (number)			Total	Allelic frequencies		χ^2 (HW)	P (HW)
	GG	GA	AA		G	A		
GZ	0.9181(258)	0.0534(15)	0.0285(8)	281	0.9448	0.0552	66.89	0.0000
SN	0.9366(251)	0.0373(10)	0.0261(7)	268	0.9552	0.0448	85.19	0.0000
Total	0.9271(509)	0.0455(25)	0.0273(15)	549	0.9499	0.0501	149.30	0.0000

HW = Hardy-Weinberg; GZ = Guanzhong breed; SN = Xinong Saanen breed.

Gene heterozygosity, effective allele number and PIC of goat *LF* locus in the two dairy goat breeds (GZ and SN) are 0.1042/0.0855, 1.1164/1.0935 and 0.0988/0.0819, respectively (Table 2). Generally, PIC is classified according to the following three types: low polymorphism (PIC value <0.25), median polymorphism (0.25 < PIC value < 0.5) and high polymorphism (PIC value >0.5). According to this classification of PIC, both GZ and SN were classified as low polymorphism according to the PIC evaluation.

Table 2. Population genetic indexes at the *LF* locus in two dairy goat breeds.

Breeds	Gene homozygosity	Gene heterozygosity	Effective allele numbers	PIC
GZ	0.8958	0.1042	1.1164	0.0988
SN	0.9145	0.0855	1.0935	0.0819
Total	0.9048	0.0952	1.1052	0.0906

PIC = polymorphism information content; GZ = Guanzhong breed; SN = Xinong Saanen breed.

Three body measurement traits were analyzed by comparison between the genotypes of 338 individuals (GZ, N = 138; SN, N = 200) and their phenotypic data. The results of as-

sociation analysis of the gene-specific SNP marker are shown in Table 3. At the SNP marker, there is a significant effect on the WH in the total population ($P < 0.05$), and animals with the genotype AA have higher mean values for WH than those with the genotype GG. We then analyzed phenotypic data of milk composition of 549 individuals with their genotypes (Table 4). Genotypes of the G198A SNP have a significant association with milk protein ($P < 0.05$) and acidity ($P < 0.05$), and animals with the genotype AA had higher mean values for milk protein than those with the genotype GA ($P < 0.05$). Animals with the genotype AA have higher acidity than those with genotypes GA and AA ($P < 0.05$). No significant correlation was observed between any of the marker genotypes at G198A and other milk composition and body measurement traits.

Table 3. Association of G198A SNP genotypes with body measurement traits (WH, BL, CC) in two dairy goat breeds.

Genotypes	WH	BL	CC
GG	72.14 ± 0.27[a]	80.29 ± 0.33	91.10 ± 0.28
GA	73.74 ± 1.28[ab]	82.09 ± 1.55	91.37 ± 1.31
AA	75.69 ± 1.60[b]	81.08 ± 1.93	94.18 ± 1.64
P	0.029	0.255	0.065

Data are reported as means ± SEM in cm. WH = withers height; BL = body length; CC = chest circumference. Means followed by different superscript letters in the same column differ significantly ($P < 0.05$).

Table 4. Association of G198A SNP genotypes with milk composition in two dairy goat breeds.

Genotypes	Fat (%)	Protein (%)	TS (%)	SNF (%)	Lactose (%)	Acidity
GG	3.83 ± 0.06	3.45 ± 0.03[ab]	12.52 ± 0.08	8.58 ± 0.03	3.98 ± 0.02	6.38 ± 0.07[a]
GA	3.83 ± 0.28	3.35 ± 0.13[a]	12.50 ± 0.36	8.57 ± 0.14	4.09 ± 0.08	6.25 ± 0.32[a]
AA	3.35 ± 0.36	3.76 ± 0.16[b]	12.26 ± 0.47	8.78 ± 0.18	3.85 ± 0.10	7.41 ± 0.41[b]
P	0.194	0.046	0.581	0.279	0.066	0.014

Data are reported as means ± SEM. TS = total solids; SNF = solid-non-fat. Means followed by different superscript letters in the same column differ significantly ($P < 0.05$).

DISCUSSION

Milk composition and body measurement traits are affected by many factors, such as genotype, sex, age, breed, herd, location, and other random environmental factors. But we have established a new statistical model in which three factors (breed, herd and location) were involved, and then we employed the least-squares method in GLM procedure of the SAS software to perform the related analysis. However, we did not find a significant difference ($P > 0.05$) (data not shown).

As we have pointed out, *LF* is a multifunctional protein and has lots of bioactive compounds in organism. Studies on polymorphisms of the *LF* gene have recently been conducted in both human and bovine. The SNPs of the *LF* gene, which were detected in humans, were associated with cancer (Liu et al., 2002) and diarrhea (Mohamed et al., 2007). In bovine, the SNP effects of the *LF* gene on milk production traits and mastitis were analyzed and positive results were found (Li et al., 2004). Apart from LF's antibacterial and tumor growth-inhibiting functions, its physiological roles in bone growth (Cornish, 2004) and the efficient expression of the milk protein (Masson et al., 1966) are also very important and should not be ignored. In

the present study, a genome-wide approach (PCR-SSCP, sequencing) was applied to analyze the polymorphism of the *LF* gene and its relationship with milk composition and body measurement traits in dairy goats. The present study showed primarily that the G198A SNP of the goat *LF* gene is significantly associated with withers height (Table 3) and the content of milk protein (Table 4). These results further illustrated LF's powerful bioactivity in the organism. The SNP of G198A, a synonymous mutation of arginine, results in an increase of a part of phenotypic variation, especially on the withers height and milk protein. Therefore, we speculate that the mutation for G198A may have an important influence on many other genes, which are involved in bone growth and the content of milk protein.

In conclusion, one SNP has been identified in the *LF* gene and its association with milk composition and body measurement traits in two breeds of dairy goat was investigated in the present research. Our results provide evidence that the *LF* gene might have potential effects on milk composition and body measurement traits in goat. Therefore, further study using the SNP for marker-assisted selection in larger populations will be necessary. It is also important to investigate whether the *LF* gene plays a role in the development of those traits and whether it involves linkage disequilibrium with other causative mutations.

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