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Polymorphisms of *PRLR* and *FOLR1* genes and association with milk production traits in goats

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ABSTRACT. We investigated the polymorphisms of *PRLR* and *FOLR1* genes in Xinong Saanen, Guanzhong, and Boer goat breeds by DNA sequencing and PCR–RFLP. Two novel SNPs were identified: KC109741: g.62130C>T in the 3'-UTR of goat gene *PRLR*, and KC136296: g.7884A>C in exon 3 of goat gene *FOLR1*. In the three goat breeds, the polymorphism information content was 0.20-0.27 at the *g*.62130*C>T* locus. At the *g*.7884*A>C* locus, it was 0.36 in Boer goats. The three goat breeds were in Hardy-Weinberg disequilibrium at the *g*.62130*C>T* locus. The g.62130C>T SNP was found to be significantly associated with milk production traits in Xinong Saanen and Guanzhong breeds. These results are consistent with the regulatory function of PRLR in mammary gland development, milk secretion, and expression of milk protein genes; they extend the spectrum of genetic variation of the goat *PRLR* gene, which could be useful for breeding programs.

Key words: SNP; PCR-RFLP; *PRLR*; *FOLR1*; Genotype

INTRODUCTION

Prolactin receptor (PRLR) belongs to the same family as the growth hormone receptor and is part of the cytokine receptor superfamily, characterized by its ability to activate JAK2 and three members of the Stat family, Statl, Stat3, and Stat5 (Clevenger et al., 1998). There are two distinct PRLR isoforms, produced by alternative splicing of the primary transcript: the long isoform has 581 amino acids and the short one has 296 amino acids (Viitala et al., 2006). The short form is unable to mediate transcriptional activation via the JAK2/STATs pathway, which inhibits the long form-mediated activation of JAK2 and transcription by the formation of heterodimers (Bole-Feysot et al., 1998; Clevenger and Kline, 2001). The long isoform of PRLR binds prolactin and contributes to the activation of JAK2 kinases and subsequent phosphorylation of STAT5 transcription factors, which bind to recognition sequences located in promoters of milk protein genes (Bole-Feysot et al., 1998). From an animal breeding perspective, polymorphisms of the *PRLR* gene have been associated with fiber and reproductive traits in goats, pigs and sheep (Terman, 2005; Chu et al., 2007; Zhou et al., 2011), as well as with milk production traits in dairy cattle (Iso-Touru et al., 2009). The *PRLR* gene has been mapped on bovine and goat chromosome 20 (Hayes et al., 1996). A segregating quantitative trait locus (QTL) for milk yield traits on bovine chromosome 20 has been found in a few dairy breeds (Fontanesi et al., 2007). The folate receptor (FOLR), also known as the folic acid binding protein, binds 5-methyltetrahydrofolate (5-MeTHF) with high affinity (Tian et al., 2012). The folate receptor gene family includes four members (FOLR1, FOLR2, FOLR3, and FOLR4), whose encoded products bind folic acid with high affinity (Leamon and Jackman, 2008). FOLR1 and FOLR2 encode glycosyl phosphatidylinositol-anchored endocytic receptors found to be expressed in some epithelial tissues (Ross et al., 1994). Folate receptor α (FOLR1) has shown the greatest change in gene expression in three species [12.7-fold in cow (N = 3), 15.4-fold in fur seal (N = 1), and 2.4-fold in tammar (N = 4)] during periods of increased milk protein production (Menzies et al., 2009), agreeing with previous reports that folate is important for milk protein synthesis and suggesting FOLR1 as a key regulator of folate metabolism for milk protein synthesis in the mammary gland. Advances in this knowledge not only give us insight into milk production in dairy goats but also provides helpful information to dissect the candidate genes in *Capra hircus* and improve milk production, in quantity and quality, through genetic manipulation. On the basis of the above considerations, we detected the polymorphisms of caprine *PRLR* and *FOLR1* genes in three goat breeds and investigated the associations between genetic marker and milk production traits. This study provides some useful information on goat genetic resources and breeding.

MATERIAL AND METHODS

DNA samples and milk traits

Genomic DNA samples were obtained from 711 goats belonging to three breeds: Xinong Saanen goat (SN; N = 323), Guanzhong goat (GZ, N =197), and Boer goat (BG; N =191). They were reared in Qianyang, Zhouzhi, and Liuyou counties of Shaanxi Province, respectively. All diets were based on alfalfa, corn silage, and a combination of concentrates including corn, soya meal, and bone meal. Health, fertility, and production records were maintained by

the dairymen and veterinarians. Milk yields from first to third lactation were standardized to 300 days in milk. For milk analysis, a milk sample was taken from each animal once a month throughout lactation, sampling at least 20 days after parturition to exclude the risk of contamination with colostrum. Goats were milked twice a day at constant intervals and a 10-mL sample from each milking session was mixed for the analysis. Milk constituents (protein and fat) were determined with an ultrasonic S60SEC milk analyzer (Milkotronic Company, Nova Zagora, Bulgaria). Five milliliters blood per goat were collected aseptically from the jugular vein and kept in a tube containing ACD anticoagulant (citric acid:sodium citrate:dextrose - 10: 27: 38). All samples were sent to the laboratory in an ice box. The genomic DNA was extracted from white blood cells using the standard phenol-chloroform extraction protocol (Santos et al., 2010; Ghaffariyan et al., 2012). All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Polymerase chain reaction (PCR) amplification and SNP investigation

According to the bovine *PRLR* and *FOLR1* genes (GenBank accession Nos. NC_007318 and AC_000172), four pairs of primers were designed to amplify the 3'-UTR and exons 2 and 3 of goat the *PRLR* and *FOLR1* genes (Table 1). Their optimal annealing temperatures are showed in Table 1. Herein, we screened them for identifying SNPs of those genes by DNA pooling sequencing assay (Bansal et al., 2002). Five microliters DNA (100 ng/μL) per sample were collected to create a DNA pool for each goat breed. PCR products were sent to Beijing Genomics Institute (Beijing, China) to be sequenced in both directions. Discovery of SNPs was conducted using the Chromas 2.31 and the DNAstar 7.0 software.

Table 1. Primer sequences for goat *PRLR* and *FOLR1* genes applied for screening polymorphisms and genotyping.

Primer	Sequence (5'→3')	Gene region	Amplicon (bp)	Tm (°C)
PRLR-F1	AGAAGAACCAGCCAAGAAG	3'-UTR	349	52
PRLR-R1	CACACAAGATGGAACGATTT			
PRLR-F2	AGTGAGAGTTATGGAAGGATG	3'-UTR	443	55
PRLR-R2	AAGGTTAAGCAACTGGTCTT			
FOLR1-F1	CCAGGAAGGTATTGTCTCAA	Exon 2	499	58
FOLR1-R1	GCTCTAGGCATTGTTCTGA			
FOLR1-F2	GTTCCTCCACCTGATGTT	Exon 3	414	54
FOLR1-R2	CCTCCTCAGACCAGAATT			

The SNPs of the *PRLR* and *FOLR1* genes were genotyped by PCR-restriction fragment length polymorphism (RFLP). The 25-μL volume contained 50 ng genomic DNA, 12.5 μL 2X reaction mix (including 500 μM of dNTP each; 20 mM Tris-HCl, pH 9; 100 mM KCl; 3 mM $MgCl_2$), 0.5 μM of each primer, and 0.5 U *Taq* DNA polymerase. The cycling protocol was 5 min at 95°C, 35 cycles of denaturation at 94°C for 30 s, annealing at X°C (Table 1) for 30 s and extension at 72°C for 35 s, with a final extension at 72°C for 10 min. PCR products (5 μL) of different primer pairs were mixed with 0.7 μL 10X buffer, 2.5 U restriction enzyme (NEB, Ipswich, UK) and 3.8 μL sterilized ddH_2O, and then incubated for 1.5 h at 37°C. The restriction enzymes are shown in Table 2. Digestion products were subjected to horizontal 3.5% agarose gel electrophoresis. The agarose gels were stained with ethidium bromide, and the genotypes were then determined.

Analysis of microRNA sites

The effects of allele substitution on microRNA binding sites were analyzed using the MicroInspector software (Rusinov et al., 2005).

Statistical analysis

The allelic frequencies, heterozygosity (H_E) and the polymorphism information content (PIC) were calculated using Popgene (version 1.31). Association analyses between *PRLR* genotypes and dairy traits were performed with the SPSS 16.0 software. Multiple comparisons of the means were performed using the least significant difference method. Data were analyzed with the following mixed linear model for SNPs and traits: $Y_{ikm} = \mu + G_i + N_K + E_{ikm}$, where Y_{ikm} is the trait measured on each of the ikmth animal, μ is the overall population mean, G_i is the fixed effect associated with the ith genotype, N_k is the fixed effect associated with kth number of kids born, and E_{ikm} is the random error. Effects associated with farm, birth year, and season of birth are not matched in the linear model, since the preliminary statistical analyses indicated that these effects did not have a significant influence on variability of traits in the populations analyzed.

RESULTS

Polymorphisms of goat *PRLR* and *FOLR1* genes

Sequencing of the amplicons of different primer pairs identified two polymorphic nucleotide sites in the goat *PRLR* and *FOLR1* genes (Figures 1 and 2). The PCR product of the PRLR-F2 and PRLR-R2 primer pair had a g.62130C>T mutation, which was in the 3'UTR of the goat *PRLR* gene (GenBank accession No. KC109741). The g.7884A>C synonymous mutation was in exon 3 of the goat *FOLR1* gene (GenBank accession No. KC136296), which was only found in BG goats. The bands of different genotypes are shown in Figures 3 and 4. In the three goat breeds, PIC was 0.20-0.27 at the *g*.62130*C>T* locus (Table 2). At the *g*.7884*A>C* locus, PIC was 0.36 in BG goats (Table 2). The genotypic distribution and allelic frequencies of the two SNPs are shown in Table 2. It was shown that the three goat breeds were in Hardy-Weinberg disequilibrium at the *g*.62130*C>T* locus ($P < 0.05$) (Table 2).

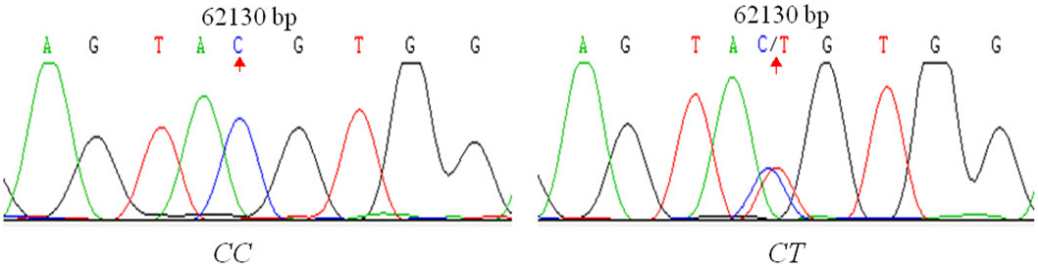

Figure 1. Sequencing maps of g.62130C>T SNP in the 3'-UTR of goat *PRLR* gene.

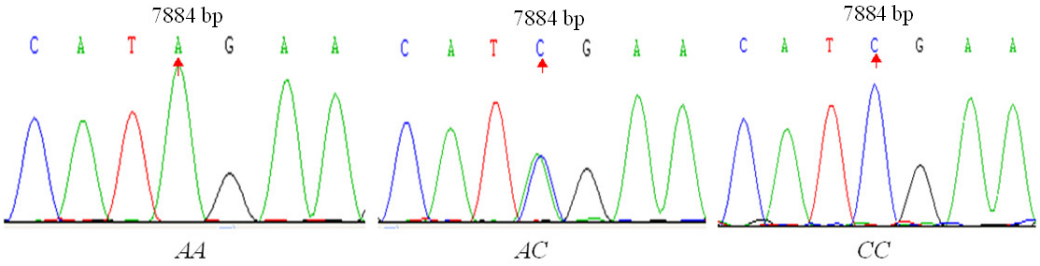

Figure 2. Sequencing maps of g.7884A>C SNP in the exon 3 of goat *FOLR1* gene.

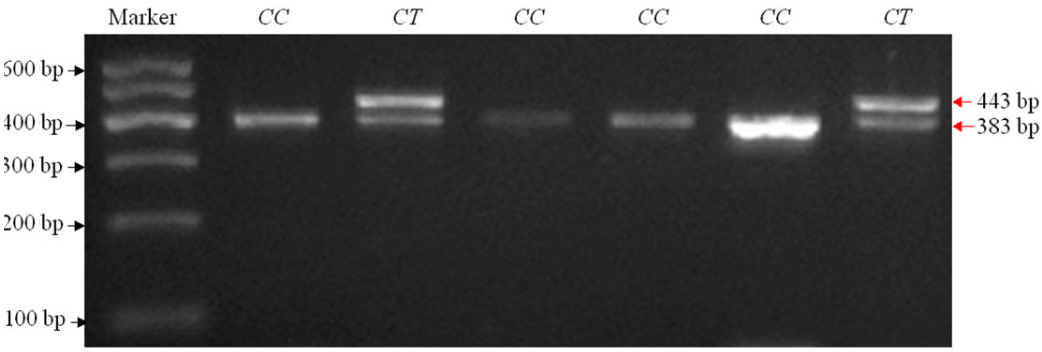

Figure 3. Electrophoresis patterns obtained after digestion with *Rsa*I endonuclease at *g*.62130*C*>*T* locus. Fragments including 60 bp of CC and CT genotypes were invisible.

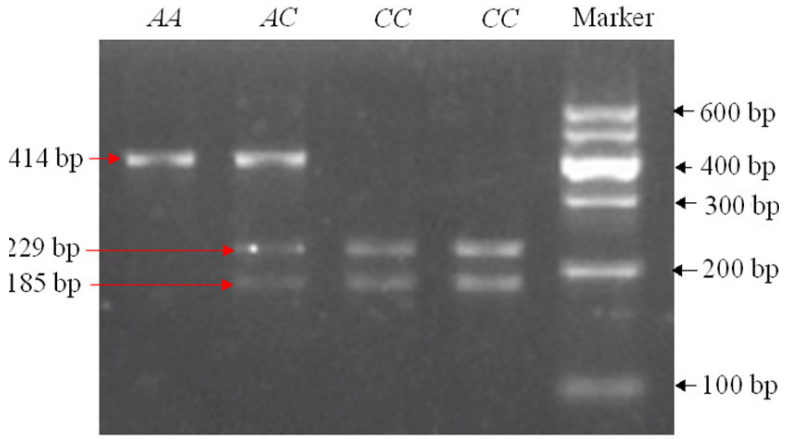

Figure 4. Electrophoresis patterns obtained after digestion with *Sfa*NI endonuclease at *g*.7884*A*>*C* locus.

Association analysis of SNP with milk production traits

In the SN breed, individuals with the *CC* genotype had greater milk yield than those with the *CT* genotype in the first, third, and average lactation at the *g*.62130*C*>*T* locus ($P < 0.05$) (Table 3); in the GZ breed, individuals with *CC* genotype had higher milk yield than those

with the *CT* genotype in the third and average lactation at the *g*.62130*C*>*T* locus (P < 0.05).

Effects of allele substitution on microRNA sites

The g.62130C>T SNP in the 3'UTR of *PRLR* gene associated with production traits was analyzed for its potential effects on micro(mi)RNA binding sites. Analysis of miRNA sites *in silico* predicted that the g.62130C>T SNP affects miRNA binding (Table 4). Both alleles of the g.62130C>T SNP introduced several different miRNA sites, whereas the C allele introduced a miRNA binding site that was abrogated in the presence of the T allele.

Table 2. Genotypic distribution and allelic frequencies of two SNP loci in *PRLR* and *FOLR1*genes.

Locus	Restriction enzyme			Breed		
				SN	GZ	BG
g.62130*C*>*T*	*Rsa*I	Genotype	*CC*	215	145	112
			CT	108	52	79
		Allele	*C*	0.83	0.87	0.79
			T	0.17	0.13	0.21
		H_E		0.33	0.26	0.41
		PIC		0.24	0.20	0.27
		Equilibrium χ^2 test		P < 0.01	P = 0.03	P < 0.01
g.7884*A*>*C*	*Sfa*NI	Genotype	*CC*	323	197	59
			AC	-	-	119
			AA	-	-	13
		Allele	*C*	1	1	0.62
			A	0	0	0.38
		H_E		-	-	0.62
		PIC		-	-	0.36
		Equilibrium χ^2 test		-	-	P < 0.01

Table 3. Association analysis of *g*.62130*C*>*T* locus with milk yield and constituents (means ± standard errors) in goats.

Breed	Genotype	Number	First lactation milk yield (kg)	Second lactation milk yield (kg)	Third lactation milk yield (kg)	Average milk yield (kg)	Milk fat (%)	Milk protein (%)
SN	*CC*	200	583.30 ± 13.25[b]	663.46 ± 6.31	783.53 ± 7.33[b]	678.83 ± 6.80[b]	3.28 ± 0.05	2.96 ± 0.01
	CT	105	567.25 ± 13.48[a]	650.97 ± 8.22	762.28 ± 9.26[a]	663.32 ± 8.27[a]	3.32 ± 0.07	2.97 ± 0.01
GZ	*CC*	145	553.44 ± 4.02	656.30 ± 21.21	758.62 ± 9.67[b]	647.16 ± 6.94[b]	3.46 ± 0.03	3.03 ± 0.01
	CT	46	543.55 ± 7.06	641.23 ± 22.93	725.78 ± 12.75[a]	630.05 ± 9.29[a]	3.49 ± 0.05	3.00 ± 0.02

Values with different superscripts within the same column in particular population differ significantly at P < 0.05.

Table 4 Effects of *PRLR* 3'-untranslated region SNP on microRNA binding sites.

SNP	Allele	miRNA name	miRNA sequence	Free energy ΔG (kcal/mol)
g.62130C>T	C	bta-miR-2317	cucugugaugacaauaccugaua	-21.6
	C	bta-miR-2324	ugggguuggggcaguguggcugu	-24.1
	C	bta-miR-302a	aagugcuuccauguuuuaguga	-20.6
	T	bta-miR-2317	cucugugaugacaauaccugaua	-21.6
	T	bta-miR-2324	ugggguuggggcaguguggcugu	-24.1

DISCUSSION

The *g*.62130*C*>*T* and *g*.7884*A*>*C* loci were in Hardy-Weinberg disequilibrium (P < 0.05), which showed that the genotypic frequencies had been affected by selection, muta-

tion or migration. According to the classification of PIC (low polymorphism if PIC < 0.25, moderate polymorphism if 0.25 < PIC < 0.50, and high polymorphism if PIC > 0.50), the SN and GZ breeds at the *g*.62130*C*>*T* locus had low genetic diversity. The BG breed at the *g*.7884*A*>*C* locus had moderate genetic diversity. The identification of candidate genes that are responsible for variation in continuous traits or quantitative traits has been a challenge in modern genetics. So far, there have been some studies of the *PRLR* gene as a candidate gene in milk production traits in animals (Viitala et al., 2006; Lü et al., 2011a). Brym et al. (2005) reported that one SNP (A→C) was found in the 205 nucleotide position of *PRLR* intron 9 for Jersey and Polish Black-and-White cattle, respectively, and that Jersey cows of CC genotype produced more milk with higher protein content than those of *AA* and *AC* genotypes. Zidi et al. (2010) found that G401R (c.1201G>A) and T452I (c.1355C>T) amino acid substitutions were associated with milk composition traits in a Murciano-Granadina goat population. Previous studies in dairy cattle demonstrated six amino acid substitutions within the signal peptide and extracellular domains of PRLR, including two polymorphisms (K2F and S18N) in exon 3 and four (L186P, S208C, R225Q, and L226I) in exon 7 (Viitala et al., 2006; Scotti et al., 2007; Zhang et al., 2008), which were significantly associated with milk production traits in Finnish Ayrshire and Holstein cattle breeds. Two newly detected SNPs (g.9206G>A and g.9681C>T) caused amino acid variations E378K and A536V, respectively, which were significantly associated with milk yields, and cows with the combined genotype *GGCC* showed superior milk performance (Lü et al., 2011b). In this study, the *g*.62130*C*>*T* SNP was located in the 3'-UTR, but the results showed that it was associated with milk yield in the SN and GZ breeds. The reason could be that the C allele at the *g*.62130*C*>*T* locus introduced a miRNA site for bta-miR-302a. Limited information is available on the effects of the introduction or abrogation of miRNA binding sites on genes involved in growth, postnatal development, and production traits in cattle. However, miRNA are well established as posttranscriptional gene expression regulators and are thought to regulate as many as 5300 human genes (Lewis et al., 2005). The biochemical and physiological functions, together with the results obtained in our study, indicate that the SNP (g.62130C>T) associated with milk performance has potential applications in a marker-assisted selection program for dairy goat breeding.

CONCLUSIONS

The g.62130C>T SNP in the goat *PRLR* gene had significant effects on milk production traits. In addition, the present study showed it to be a genetic marker for goat genetics and breeding, with potential applications in breeding programs.

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