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DNA METHYLATION PATTERN OF ESTROGEN RECEPTOR ALPHA (*ERA*) GENE PROMOTER REGION AND ITS INFLUENCE ON MILK PRODUCTION PERFORMANCE IN XINONG SAANEN DAIRY GOATS

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ABSTRACT

The aim of this study was to identify DNA methylation pattern of estrogen receptor alpha *(ERα)* gene promoter region and to evaluate its association with milk production performance of Xinong Saanen dairy goats. The results showed that there was no significant difference in DNA methylation percentage (MP) in mammary glands samples collected during dry period and milking period. Interestingly, the methylation of *ERα* gene was breed-specific, and the high milk yielding breed of Xinong Saanen dairy goats appeared to be linked with hypomethylation of the gene ($P<0.001$) when compared with other dairy breeds. Methylation share rate (MSR) of Xinong Saanen dairy goats ranged from 0 to 0.105. Furthermore, the DNA MP in whole blood samples was significantly higher than that in mammary gland ($P<0.001$), suggesting that the methylation of this gene was tissue-specific. For the average milk density (AMD), the DNA methylation level of *ERα* gene in junior AMD group (JP) was significantly lower than that in senior AMD group (SP) ($P<0.05$). The MP of CpG-12 and CpG-15 dinucleotide loci of SP group were significantly higher than those of JP group ($P<0.05$ and $P<0.05$), respectively. Therefore, the two specific CpG-dinucleotide loci can be considered as epigenetic markers, which may contribute in improving milk production performance through epigenetic marker-assisted selection (eMAS) in dairy goats.

Key words: Dairy goat; Estrogen receptor alpha (*ERα*) gene; DNA methylation; Milk production performance; Influence.

INTRODUCTION

Presently, dairy products are important nutritional necessities in human life, and most of them are processed from ruminant milk (especially that of cow and goat) containing specific bioactive proteins, lipids, saccharides, immunoglobulins, enzymes, antimicrobial peptides, oligosaccharides, hormones, cytokines and growth factors (Donovan, 2006; Pouliot and Gauthier, 2006; Zervas and Tsiplakou, 2013). Although, the protein fractions of ruminant milk are qualitatively very similar (Vargas *et al.,* 2008), but, in comparison with bovine milk, dairy goat milk is more advantageous in terms of being more digestible and more effectively triggering innate and adaptive immune responses in human system (Chen, 2009; Ceballos *et al*., 2009; Li *et al*., 2013; Jirillo and Magrone, 2014). However, the breed scale and the milk yield of dairy goat are considerably lower than that of dairy cow. Therefore, the studies pertaining to milk production performance of dairy goat needs to be emphasized.

Numerous studies have found that estrogen plays an important role in regulating the growth of mammary glands and lactation, effect mediated through its receptors (Athie *et al*., 1996; Bocchinfuso and Korach, 1997). To date, it is known that estrogen receptors (ER) have two subtypes: ERα and ERβ, and the former mainly mediate the effect of estrogen on mammary glands (Kuiper *et al*., 1996). The previous studies have also shown that estrogen combines with ERα and acts on responsive promoter region of the target gene to induce its transcription, indicating that ERα targets the lactation in mammals (Mourad *et al*., 2014). This reflects that ERα gene and lactation are closely related.

Latest literature indicates that there is a novel mechanism to affect the expression of genes without any mutation in nucleotide sequence of DNA, called epigenetic modification (Egger *et al*., 2004; Miranda and Jones, 2007; Illingworth and Bird, 2009; Sharma *et al*., 2010; Ghavifekr *et al*., 2013). As a major kind of epigenetic modification (Egger *et al*., 2004), methylated DNA is formed by a biochemical process in which a methyl group provided by S-adenosylmethionine is added to 5' position of cytosine. Generally, DNA methylation occurs in the regions named "CpG islands" which are having a high frequency of CpG-dinucleotides, and they often encompass promoters of many vertebrate genes (Illingworth and Bird, 2009). Meanwhile, DNA methylation is affected by multiple factors, such as temperature, nutrients, early environmental stimulation,

radiation, and abnormal chemical factors (Dubrova, 2003; Weaver *et al*., 2004; Baccarelli and Bollati, 2009; Chinnusamy and Zhu, 2009; Widiker *et al*., 2010). Methylated DNA has various characteristics such as it is hereditable, stable and can convey abundant genetic information (Wolf *et al*., 1984; Cooper and Youssoufian, 1988; Laird, 2003). Interestingly, DNA methylation profile undergoes heavy programming in the early stages of embryonic development, which remains stable during adult stage and regulates gene expression steadily (Messerschmidt *et al*., 2014). As the cell divides, DNA methylation status can be inherited, so the progeny DNA has the same methylation profile. Therefore, these many features of DNA methylation indicate its applicability as a novel epigenetic marker for molecular breeding.

As from above, *ERα* gene plays an important role during lactation in dairy goats. However, methylation pattern of this gene, having a significant influence on lactation traits, is rarely reported in dairy goats except by Rong *et al.,* (2011). As a famous dairy breed, the Xinong Saanen dairy goat is reared mainly in Northwest and Southeast China. However, its milk production performance is decreasing and it cannot adapt to the ongoing developments in Chinese dairy goat industry. So, the epigenetic marker-assisted selection (eMAS) can be implemented to lift milk production performance of this breed by overcoming its negative traits (da Costa *et al*., 2014). Hence, the aim of this work is to explore DNA methylation status of *ERα* gene promoter region in mammary gland and blood of Xinong Saanen dairy goat, as well as to evaluate its association with milk production performance. The results of our study provide useful DNA methylation markers for enhancing lactation performance in Chinese dairy goat industry.

MATERIALS AND METHODS

Sample collections and classification of the Xinong Saanen dairy goats: The study was conducted in compliance with the requirements of the Animal Ethics Committee of Northwest A&F University. According to the experimental protocols, the collected samples were divided into mammary gland tissue samples and blood samples.

Collection of mammary gland tissues:A total of 4 randomly selected healthy female Xinong Saanen dairy goats were obtained from Yangling High-Tech Agriculture Demonstration District, Shaanxi province, P. R. China. They were housed in warm cots that were kept ventilated and dry, where they could eat and drink freely. According to the phase of the adult goat's mammary gland development, i.e. whether they are in state of lactation or involution, they were divided into two periods: milking period (postpartum 15 days to 6 months) (two parallel groups: M1 and M2) and dry period (15 days after stopping milking) (two parallel groups: D1 and D2) (Li *et al*., 2012). Fresh mammary gland tissues were collected and stored in liquid nitrogen quickly for 2-3 days, and kept in -80^{o}C freezer for long time.

Collection and classification of blood groups: To exclude the age-specific differences in DNA methylation pattern of blood, blood samples were obtained from 268 healthy and unrelated three-year-old lactating female Xinong Saanen dairy goats from the dairy goat breeding farms in Qianyang county and Yangling High-Tech Agriculture Demonstration District, Shaanxi province, P.R. China. Based on our previous descriptions (Lan *et al*., 2013a; Zhao *et al*., 2013a), average milk density (AMD) was measured by MilkoScan FT120 (FOSS Corporation, Denmark). According to the statistical probability (95.00%) of beyond 1.96 standard deviations (SD) for AMD, referring to the published literature (Pan *et al*., 2013), animals were classified and divided into two groups according to their AMD values: low AMD group and high AMD group. According to Student's t-test, low group of AMD was significantly lower than average; at the same time, high group was significantly higher than average. Moreover, there was a significant difference between low and high group in the result of statistical analysis. Then, these samples, namely, J1 (Junior milk density group 1), J2 (Junior milk density group 2), S1 (Senior milk density group 1), and S2 (Senior milk density 2), were used for this study.

Prediction of the CpG island and methylation primer design: Since goat *ERα* gene sequence was not available, the bovine *ERα* gene sequence (NCBI Gene ID:37223522) was referred to amplify the 352 bp length fragment covering the CpG island (including 16 CpG-dinucleotide loci) at 5'-flanking region within goat *ERα* gene (Figure 1). A pair of primers [Forward methylation primer: 5'GAGATAAATA GAGATAGATTGG3' (22 nt) and reverse methylation primer: 5'AATCTCCAACTTTAAATACAACT3' (23 nt)], located between 722 nt-1073 nt, were designed using Methprimer (http://www.urogene.org/methprimer/).

Genomic DNA extraction and bisulfate treatment: Genomic DNA samples were isolated from mammary gland tissues and blood samples collected from all of the chosen individuals according to the procedure described previously (Pan *et al*., 2013). All DNA samples were measured and diluted to the final concentration of 50 ng/μL. The qualified DNA samples from the low and high groups were equally selected to construct genomic DNA pools. Each pool containing 1000 ng mixed with genomic DNA was processed by sodium bisulfite using QIAGEN Epitect fast DNA Bisulfite kit (QIAGEN, Germany) following the manufacturer's instructions.

PCR amplification: The PCR amplification was performed in 25 µL volume which contained 50 ng bisulfite-processed pooled DNA, 0.5 µM of each primer, 1 × buffer (including 1.5 mM $MgCL_2$), 200 µM dNTPs and 0.625 units of *Taq* DNA polymerase (MBI, Vilnius, Lithuania). Touch-down PCR program was used to carry out DNA amplification in the S1000 Thermal Cycler (Bio-Rad, USA): 5 min at 95 ºC; 2 cycles of 94 ºC for 30 s, annealing from 68 ºC to 52 ºC by 2 ºC decrease for 30 s respectively, 72 ºC for 30 s; 30 cycles of 94 ºC for 30 s, 50 ºC annealing for 30 s, 72 ºC for 30 s; a final extension at 72 ºC for 10 min; subsequently cooling to 4 ºC. The total of 25 µL PCR products was gel-purified using the EasyPure Quick Gel Extraction Kit (TransGene Biotech, Beijing, China). The purified fragments were inserted into the pGEM-T easy vector (Promega, USA). The colony PCR was used to verify the positive colonies and those were sequenced via sequencing service (Genscript, Nanjing, China). In terms of transformation efficiency, the number of positive colonies for each pool that was sent for sequencing should range from 15 to 25.

Statistical analysis

Population genetics indices for DNA methylation patterns: For effectual genetic analysis of methylation pattern, DNA methylation or unmethylation in different CpG dinucleotide loci was scored as 1/0. Locus methylated frequencies (L-MF), observed methylation CpG-dinucleotide numbers (OMCN), observed polymorphic methylation CpG dinucleotide numbers (OPMCN), and methylated percentages (MP) were directly calculated. Additionally, as a measure of epigenetic diversity, genetic indices including locus heterozygosity (L-He), locus homozygosity (L-Ho), and genetic diversity index (H) were calculated by Nei's method. The most important of these indices is Shannon's genetic diversity index (H), calculated using the formula $H=-\sum\pi_{i*}\ln\pi_{i,}$ where π_i stands for the frequency of each amplified methylation pattern in the general population. Methylated share rates (MSR) was also calculated as described by Pan *et al.*, (2013).

Correlation analysis between DNA methylation pattern and lactation performance: Methylation status of each CpG-dinucleotide locus of the CpG island was obtained by sequence result, and alignment analysis using BioXM software (version 2.6.0, developed by College of Agriculture, Nanjing Agricultural University). Fisher's exact test (χ2-test) was used to correlate methylation differences at each CpG-dinucleotide locus and the entire CpG island between the different groups of blood and mammary gland tissue samples collected from Xinong Saanen dairy goat (Lan *et al*., 2013b). According to the publication in Laoshan dairy goat (Rong *et al.*, 2011), methylation differences of the *ERa* gene promoter were also analyzed between the Xinong Saanen dairy goat and Laoshan dairy goat during lactation and dry periods.

Prediction of transcription factor binding to the CpG island: In order to predict the possible transcription factors which probably combine to some CpG-dinucleotide loci, authorized and reliable online bioinformatics software was used: the MatInspector database in Genomatix (http://www.genomatix.de).

RESULTS

DNA methylation pattern of the *ERα* gene promoter: A total of 16 CpG-dinucleotide loci were figured out in the CpG island of *ERα* gene promoter (352 bp) (Figure 1 and Figure 2-A). Further, Figure 2-B and Figure 3 clearly show bisulfite sequencing and DNA methylation patterns, respectively. This study focused on the methylation status of these 16 CpG-dinucleotide loci in *ERα* gene promoter.

Genetic diversity analysis of DNA methylation patterns of *ERα* gene promoter in two breeds of dairy goats: The detailed information including L-MF, L-Ho, L-He and locus Shannon's genetic diversity index (L-H) in different CpG-dinucleotide loci of goat *ERα* gene promoter region were shown in table 1. In mammary gland of the Xinong Saanen dairy goats, the L-MF of different CpG-dinucleotide loci of individuals distributed from 0 to 0.133. In blood of the Xinong Saanen dairy goat, methylated percentages of locus of individuals ranged from 0 to 0.333. In mammary gland of Laoshan dairy goats, L-MF of locus of individuals ranged from 0 to 0.900 (Suppl. Table 1)

From table 2, there were 16 polymorphic methylation numbers in the total of all expected 16 CpG-dinucleotide loci. The numbers of unmethylation of Saanen dairy goats for different CpG-dinucleotide loci ranged from 2 to 15. The average of methylation number in Saanen dairy goat ranged from 0.08 to 2.07 and that in Laoshan dairy goat ranged from 0 to 1.70. The range of methylation percentage (MP) varied from 0.52% to 12.92%. Meanwhile, the number of unmethylation of Laoshan dairy goat for different CpG-dinucleotide loci ranged from 6 to 16. The range of mean methylated percentages varied from 0 to 10.63%. Hence, the average of MP for Xinong Saanen dairy goat was 6.0%, while that of Laoshan dairy goat was 5.6%.

In Table 3, the average heterozygote of Xinong Saanen dairy goat ranged from 0.010 to 0.225, while average H waved from 0.03 to 0.38. MSRs for different individual ranged from 0 to 0.105. The wave of maximum of MSR was from 0 to 1.000. At the same time, the average heterozygote of Laoshan dairy goat distributed from 0 to 0.190, while average H varied from 0 to 0.34. MSRs for different individuals ranged from 0 to 0.618. The scope of maximum of MSR was from 0 to

Table 1. DNA methylation patterns in different CpG loci of goat *ERα* gene 5' region for Xinong Saanen dairy goat

Sample	Types	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
M1	L-MF	0	0.056	0	0	0	0	0	0	0	0.056	0.056	0	0	0	0	0.056
	L-Ho	1.000	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	0.895	1.000	1.000	1.000	1.000	0.895
	L-He	0	0.105	0	0	0	0	0	0	0	0.105	0.105	0	0	0	0	0.105
	L-H	0	0.215	0	0	0	0	0	0	0	0.215	0.215	0	0	0	0	0.215
M2	L-MF	0.133	0.067	0	0.067	0	0	0.067	0	0.067	0	0	0	0	0	0	0
	L-Ho	0.769	0.876	1.000	0.876	1.000	1.000	0.876	1.000	0.876	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	L-He	0.231	0.124	0	0.124	0	0	0.124	0	0.124	0	0	0	0	0	0	0
	L-H	0.393	0.245	0	0.245	0	0	0.245	0	0.245	0	0	0	0	0	0	0
D1	L-MF	0	0	0.045	0.091	0	0	0	0	0	0	0.045	0.045	0.045	0	0.045	0.045
	L-Ho	1.000	1.000	0.913	0.835	1.000	1.000	1.000	1.000	1.000	1.000	0.913	0.913	0.913	1.000	0.913	0.913
	L-He	0	0	0.087	0.165	0	0	0	0	0	0	0.087	0.087	0.087	0	0.087	0.087
	L-H	0	0	0.185	0.305	0	0	0	0	0	0	0.185	0.185	0.185	0	0.185	0.185
D2	L-MF	0	0	0	0.083	0	0	0	0	0	0	0	0	0	0	0	0
	L-Ho	1.000	1.000	1.000	0.847	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	L-He	0	0	0	0.153	0	0	0	0	0	0	0	0	0	0	0	0
	L-H	0	0	0	0.287	0	0	0	0	0	0	0	0	0	0	0	0
J1	L-MF	0.133	0.133	0.067	0.067	0.067	0	0.067	0.067	0.133	0.067	0.133	0.067	0.067	0	0	0.067
	L-Ho	0.769	0.769	0.876	0.876	0.876	1.000	0.876	0.876	0.769	0.876	0.769	0.876	0.876	1.000	1.000	0.876
	L-He	0.231	0.231	0.124	0.124	0.124	0	0.124	0.124	0.231	0.124	0.231	0.124	0.124	0	0	0.124
	L-H	0.393	0.393	0.245	0.245	0.245	0	0.245	0.245	0.393	0.245	0.393	0.245	0.245	0	0	0.245
J2	L-MF	0.118	0.235	0.059	0	0.059	0.059	0.176	0	0.118	0.176	0.118	0	0.176	0.118	0	0.118
	L-Ho	0.792	0.640	0.889	1.000	0.889	0.889	0.709	1.000	0.792	0.709	0.792	1.000	0.709	0.792	1.000	0.792
	L-He	0.208	0.360	0.111	0	0.111	0.111	0.291	0	0.208	0.291	0.208	0	0.291	0.208	0	0.208
	L-H	0.362	0.546	0.224	0	0.224	0.224	0.466	0	0.362	0.466	0.362	0	0.466	0.362	0	0.362
S1	L-MF	0.227	0.136	0.273	0.045	0.091	0.091	0.136	0.045	0.091	0.182	0.045	0.136	0.136	0	0.091	0.091
	L-Ho	0.649	0.764	0.603	0.913	0.835	0.835	0.764	0.913	0.835	0.702	0.913	0.764	0.764	1.000	0.835	0.835
	L-He	0.351	0.236	0.397	0.087	0.165	0.165	0.236	0.087	0.165	0.298	0.087	0.236	0.236	0	0.165	0.165
	L-H	0.536	0.398	0.586	0.185	0.305	0.305	0.398	0.185	0.305	0.474	0.185	0.398	0.398	0	0.305	0.305
S1	L-MF	0.133	0.067	0	0.200	0.133	0.067	0.333	0.133	0	0.067	0.133	0.267	0.200	0	0.200	0.133
	L-Ho	0.769	0.876	1.000	0.680	0.769	0.876	0.556	0.769	1.000	0.876	0.769	0.609	0.680	1.000	0.680	0.769
	L-He	0.231	0.124	0	0.320	0.231	0.124	0.444	0.231	0	0.124	0.231	0.391	0.320	0	0.320	0.231
	L-H	0.393	0.245	0	0.500	0.393	0.245	0.637	0.393	0	0.245	0.393	0.580	0.500	0	0.500	0.393

[1)]M1, M2=Samples from milking period of mammary gland; D1, D2=Samples from dry period of mammary gland; J1=Junior milk density group 1; J2=Junior milk density group 2; S1=Senior milk density group 1; S2=Senior milk density 2;

[2)]L-MF=Locus methylated frequencies; L-Ho=Locus homozygote (Ho); L-He=Loucs heterozygote (He); L-H=Locus Shannon's genetic diversity index (H); [3)]C=CpG-dinucleotide number, such as C1=CpG-dinucleotide number 1;

1.000. The average of MSR for Saanen dairy goat was 0.036, as well as that of Laoshan dairy goat was 0.205.

DNA methylation comparison of each CpG-dinucleotide locus: For mammary gland samples from two groups (D and M), there was no significant difference between corresponding CpG-dinucleotide locus in each (P>0.05). However, for two different groups (J and S), it appeared different results that the Pearson test value of CpG-12 locus was 0.041 and CpG-15 locus was 0.03. Further, DNA methylation level of two CpG-dinucleotide loci (CpG-12 and CpG-15) in S group were significantly higher than that of J group (P<0.05) (Table 4) (Figure 4 A).

DNA methylation status and comparison between mammary gland of two breeds: The analysis results indicated that there was no significant methylation difference within the parallel groups (M1 vs M2; D1 vs D2) (P>0.05; P>0.05). Hence, D1 and D2 were combined as dry period group (D group), and M1 and M2 were combined as milking period group (M group). In addition, the CpG island methylation rate of D group (1.7%) was also not significantly different from M group (1.9%) (P>0.05) (Table 4). Then the data of Laoshan dairy goat mammary gland showed that the level of DNA methylation of dry period (10.63%) was not significantly different from that of milking period (10.00%) (Rong *et al*., 2011).

Therefore, it is showed that the level of DNA methylation in *ERα* gene promoter region of dry period group was not significantly different from milking period group in both two breeds of dairy goat experiment (P>0.05; P>0.05) (Table 4). Nevertheless, for the same period samples, there were significant differences between the two breeds (D: P<0.01, M: P< 0.01), indicating that the level of DNA methylation of Laoshan dairy goat was apparently higher than that of Xinong Saanen dairy goat in the same period (Figure 4 B).

DNA methylation status of blood samples of Xinong Saanen dairy goat: The MP indicated that there was no significant difference in the levels of DNA methylation during the same period (J1 vs J2; S1 vs S2) (P>0.05) (Figure 4 C). On comparing, the results of two groups of junior milk density clubbed as one (J) , and senior milk density combined group (S) which combined S1 and S2 it was found that DNA methylation level of J group (9.4%) was significantly lower than that of S group (12.0%) (P<0.05). Furthermore, there was significant difference in DNA methylation levels between blood and mammary gland during milking period (P<0.05). In details, it was revealed that degree of DNA methylation in blood was apparently higher than that of mammary gland (Figure 4 C).

Prediction of relevant transcription factors in CpG island: The result of software analysis predicted some crucial transcription factors, like zinc finger combination factors including ZF5F, SP1, KLFS, CTCF, GLIF, which can bind to some vital CpG-dinucleotide loci (Figure. 5).

Table 2. DNA metylation analysis of *ERα* gene 5' region in XinongSaanen and Laoshan dairy goat

Breed	Tissue	Sample	OMCN	Sample sizes	OPMCN	100% MCN	0% MCN	Total of MCN	Average of MCN	MP (%)	Note
Xinong	Mammary	M1	16	18	4	0	12	4	0.22	1.39	#
Saanen	Gland	M2	16	15	5	0	11	6	0.40	2.50	#
dairy		D1	16	22	7	0	9	8	0.36	2.27	#
goat		D2	16	12	1	0	15	1	0.08	0.52	#
	Blood	J1	16	15	13	0	3	17	1.13	7.08	#
	Samples	J2	16	17	12	0	4	26	1.53	9.56	#
		S1	16	22	14	0	2	40	1.82	11.36	#
		S2	16	15	13	0	3	31	2.07	12.92	#
Laoshan	Mammary	P1	16	10	3	0	13	8	0.80	5.00	##
dairy	Gland	P2	16	10	2	0	14	6	0.60	3.75	##
goat		P3	16	10	3	0	13	6	0.60	3.75	##
		P4	16	10	0	0	16	0	0	0	##
		P5	16	10	10	0	6	15	1.50	9.38	##
		P6	16	10	5	0	11	17	1.70	10.63	##
		P7	16	10	3	0	13	17	1.70	10.63	##

OMCN, observed methylation CpG-dinucleotide number; OPMCN, observed polymorphic methylation CpG-dinucleotide number; MCN, methylation CpG-dinucleotide number; MP, methylation percentage.
#These data were from this study. ##These data were from the Rong *et al.,* 2011.

Table 3. Methylated share rate (MSR) and index of epigenetic diversity of methylaton pattern of *ERα* gene 5' region.

Breed	Tissues	Sample	MSR			Average homozygote	Average heterozygote	Mean H	Note
			Mean ± SE	Min	Max				
Xinong Saanen dairy goat	Mammary Gland	M1	0 ± 0	0	0	0.973	0.027	0.07	#
		M2	0.00366 ± 0.0349	0	0.333	0.951	0.049	0.12	#
		D1	0.00289 ± 0.0439	0	0.667	0.956	0.044	0.11	#
		D2	0 ± 0	0	0	0.990	0.010	0.03	#
	Blood	J1	0.0181 ± 0.0917	0	0.500	0.868	0.132	0.26	#
		J2	0.0625 ± 0.170	0	0.800	0.827	0.173	0.32	#
		S1	0.0968 ± 0.222	0	1.000	0.799	0.201	0.35	#
		S2	0.105 ± 0.197	0	1.000	0.775	0.225	0.38	#
Laoshan dairy goat	Mammary Gland	P1	0.185 ± 0.380	0	1.000	0.905	0.095	0.20	##
		P2	0.156 ± 0.367	0	1.000	0.928	0.072	0.16	##
		P3	0.0889 ± 0.289	0	1.000	0.928	0.072	0.16	##
		P4	0 ± 0	0	0	1.000	0	0	##
		P5	0.103 ± 0.237	0	1.000	0.830	0.170	0.31	##
		P6	0.287 ± 0.341	0	1.000	0.810	0.190	0.34	##
		P7	0.618 ± 0.320	0	1.000	0.810	0.190	0.34	##

MSR, methylated share rate; SE, standard error; Min, minimum; Max, maximum; H, genetic diversity index.
[#] These data were from this study. [##]These data were from the Rong*et al.*, 2011.

Table 4 Comparisons of methylation difference at each CpG-dinucleotide locus of blood samples in Xinong Saanen dairy goats.

Tissue	Mammary gland			Blood		
	MP of D	MP of M	*P*-value	MP of J	MP of S	*P*-value
CpG-1	0.061	0	0.239	0.125	0.189	0.527
CpG-2	0.061	0	0.239	0.188	0.108	0.496
CpG-3	0	0.029	1.000	0.063	0.162	0.270
CpG-4	0.030	0.088	0.367	0.031	0.108	0.363
CpG-5	0	0	1.000	0.063	0.108	0.679
CpG-6	0	0	1.000	0.031	0.081	0.618
CpG-7	0.030	0	0.493	0.125	0.189	0.527
CpG-8	0	0	1.000	0.031	0.081	0.618
CpG-9	0.030	0	0.493	0.125	0.054	0.405
CpG-10	0.030	0	0.493	0.125	0.135	1.000
CpG-11	0.030	0.029	1.000	0.125	0.108	1.000
CpG-12	0	0.029	1.000	0.031	0.189	*0.041
CpG-13	0	0.029	1.000	0.125	0.162	0.742
CpG-14	0	0	1.000	0.063	0.054	1.000
CpG-15	0	0.029	1.000	0	0.135	*0.031
CpG-16	0.030	0.029	1.000	0.094	0.108	1.000
Average	0.017	0.019	0.820	0.084	0.123	*0.039

[1)]MP, methylation percentage;
[2)]D, dry period combined group; M, milking period combined group; J, junior milk density combined group; S, senior milk density combined group.
[3)]Average, average methylation percentage of different groups.
*indicates a significant difference between the two CpG-dinucleotide loci (***P***<0.05).

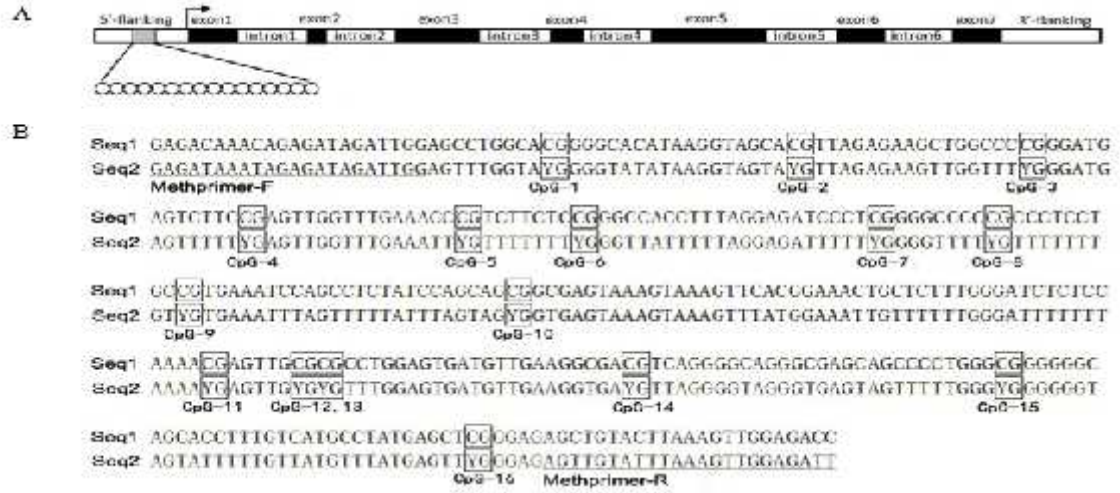

Figure 1: The structure of the CpG island and methylation primer design.
NOTE: YG, CG: methylated loci; TG (unmethylated loci); Seq1: original sequence; Seq2: the original sequence processed by sodium bisulfate; Methprimer-F: methylation forward primer; Methprimer-R: methylation reverse primer.

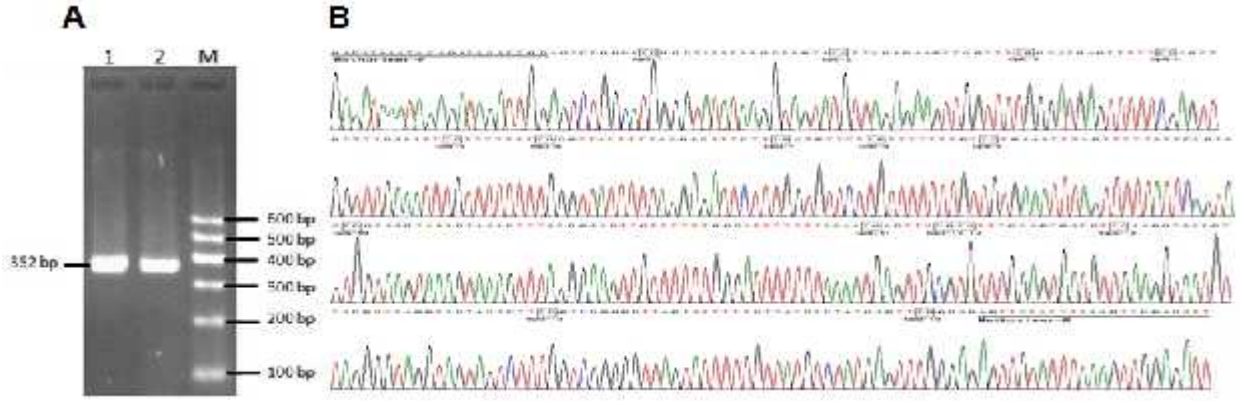

Figure 2: Analysis of electrophoresis and bisulfite sequencing of this CpGisland.

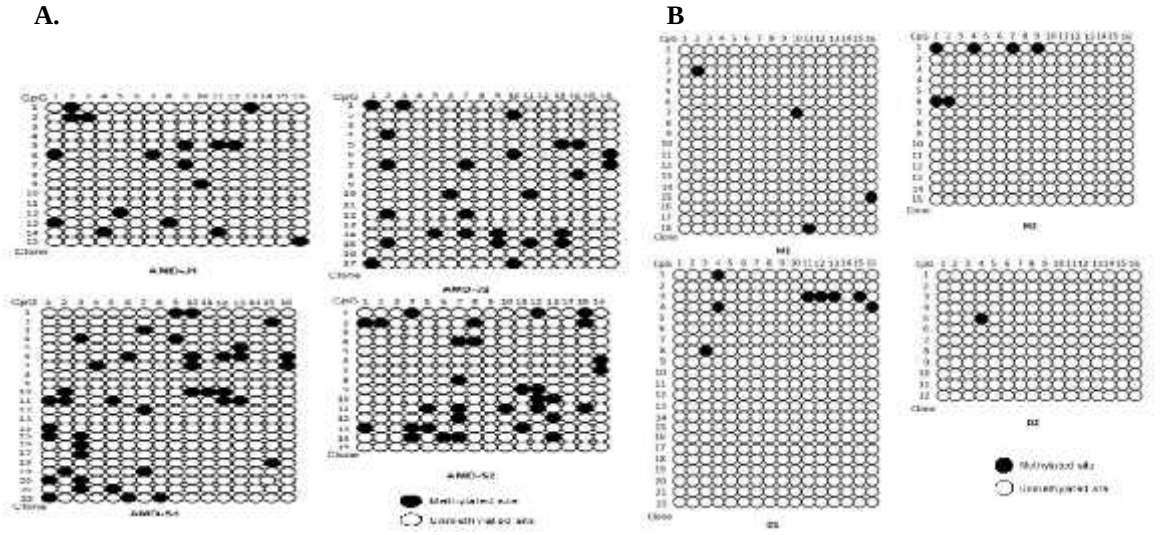

Figure 3:Methylation patterns of blood and mammary gland samples.
NOTE: AMD: average milk density; J: junior AMD group; S: senior AMD group; D: dry period; M: milking period.

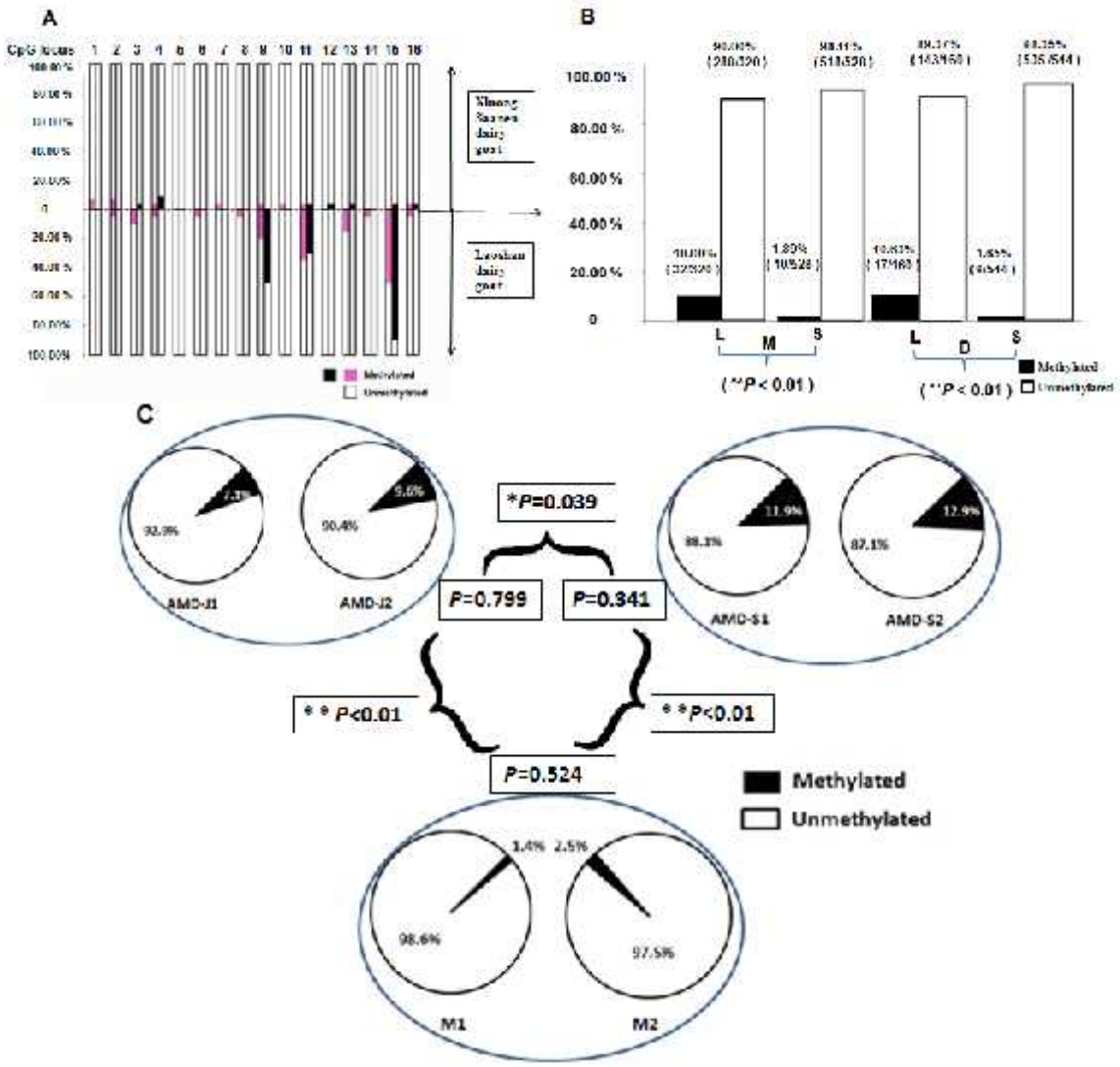

Figure 4:DNA methylation status and comparison of two breeds.

NOTE: A: methylation status of each CpG-dinucleotide of dry period and milking period of two breeds was shown in this tabulation. The left of every two combined bar chart meant methylation status of milking period; the right of every two combined bar chart meant methylation status of dry period. B: methylation status of CpG island of dry period and milking period of two breeds.C:the entire methylation percentage of blood and mammary samples from milking period of Saanen dairy goat. L, Laoshan dairy goat; S, Xinong Saanen dairy goat; M, milking period; D, dry period.

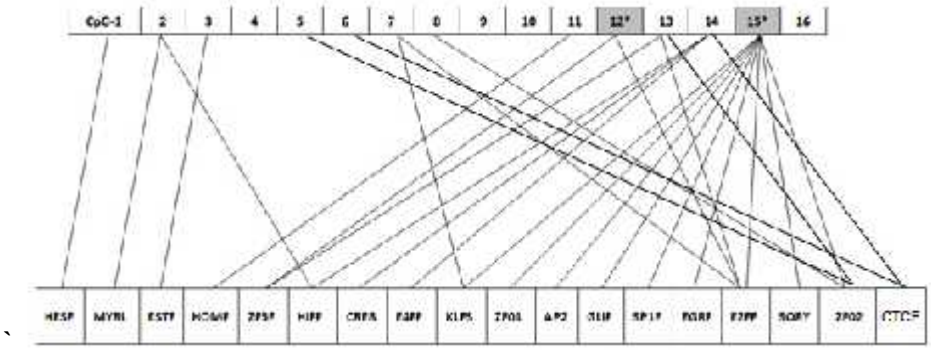

Figure 5:The crucial transcription factors predicted to bind to the CpG-dinucleotide loci of this CpG island.

DISCUSSION

As we know, DNA methylation is just like a switch in genes, which may stably alter the expression of genes without mutations in DNA coding sequences (Egger *et al*., 2004; Holliday, 2005; Huang *et al*., 2010). When CpG islands in the promoter regions are methylated abnormally, gene transcription is suppressed (Zhao *et al*., 2013b), perhaps because it influenced chromosome space conformation (Lopez-Serra and Esteller, 2008). *ERα* gene plays a crucial role during lactation; therefore, it is meaningful to study the methylation status of this gene and its association with milk production performance.

It was obvious that there was no significant difference between DNA methylation level in mammary gland tissues of dry (D) group and milking (M) group of the Xinong Saanen dairy goats, as well as of Laoshan dairy goats. However, for the same period, there appeared a significant difference between these two breeds of dairy goats. Moreover, the MP of the entire CpG island in Laoshan dairy goat was apparently higher than that in Xinong Saanen dairy goat. Furthermore, the DNA methylation profile showed a breed-specific feature. Based on genetic diversity, heterozygosity, MP and MSR, it was considered that the Xinong Saanen dairy goat possessed hypomethylation of *ERα* gene promoter, which possibly complies with high expression of *ERα* gene to have good lactation performance.

On the other hand, the results of analysis of blood samples of dairy goats were in conformity with those desired. It showed that DNA methylation of senior (S) group was significantly different from junior (J) group; meanwhile, lactation performance of two groups was also significantly different. Therefore, considering that it is easier and cheaper to collect blood samples, milk production performance of dairy goats can be forecasted on the basis of DNA methylation status of *ERα* gene promoter in blood. Further, hypermethylation of *ERα* gene promoter could be more inclined to high milk density. Hence, this can probably be used as epigenetic marker to enhance the development of molecular breeding.

It can be easily seen that DNA MP in blood samples were apparently higher than that in mammary glands, on comparing methylation status in these two samples. This finding can probably be explained on the basis that hypermethylated DNA in *ERα* gene promoter can suppress the dairy goat's milk production performance. The study also proves that the DNA methylation profile of *ERα* gene is tissue specific.

To further compare methylation differences of CpG-dinucleotide loci in *ERα* promoter region between senior (S) group and junior (J) group of Saanen dairy goat, two significantly different loci (CpG-12 and CpG-15 loci) were found. Our group predicted 18 possible important transcription factors probably linked with the whole target gene, among which, 6 possible transcription factors were potentially important. They all combine specifically with the upstream promoter element and regulate the expression by other epigenetic modifications (e.g. ZF5F, SP1F, KLFS, EGRF, and CTCF). A large number of studies have found that ZF5F (ZF5 POZ domain zinc finger), SP1F/KLFS (Specificity protein 1 and Krüppel-like factors), EGRF (Early growth response factor) may regulate the transcription of a variety of housekeeping genes with GC-rich regulatory elements in the promoter region to take part in virtually all facets of cellular function (Obata *et al*., 1999; Joanna *et al*., 2003; Matthews and Gustafsson 2003;). Meanwhile, recent evidence showed that histone acetylation and deacetylation, as one of the epigenetic modifications, might serve as a switch for SP1-like/KLF proteins to function as activators or repressors (Joanna *et al*., 2003). Therefore, this experiment indicated that hypermethylated DNA may suppress the combination of specific transcription factors to influence the formation of histone acetylation and open chromatin structure to inhibit the expression of *ERα* gene.

Briefly, this work expounded the methylation pattern of *ERα* gene promoter in mammary gland and blood, and it simultaneously uncovered two important CpG-dinucleotide loci (CpG-12 and CpG-15) with significant effects on milk density, which may have a promising application as a potential epigenetic marker. Moreover, these findings can contribute in improving milk prodution performance through epigenetic marker-assisted selection (eMAS) in dairy goats.

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