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Molecular cloning, promoter analysis, SNP detection of Clusterin gene and their associations with mastitis in Chinese Holstein cows

Zeying Wang · Jingmin Huang · Jifeng Zhong · Genlin Wang

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Abstract To examine the effect of Clusterin (CLU) on mastitis, genetic association analysis was applied on mastitis and milk production traits of 1,137 Chinese Holstein cows. We detected two novel single nucleotide polymorphisms (SNPs), G+15781A in the seventh exon and C-994T before 5′-upstream region (UTR) of *CLU* gene, found five TATA box, one CpG island and more transcription factor binding sites in promoter region, respectively, Milk fat rate in genotype AA was significantly higher than in GG on fat rate ($P < 0.01$), milk fat and milk yield in combined haplotype H1H4 (AGCT) were significantly higher than in H3H4 (GGCT) ($P < 0.05$), H1H4 was decided advantage in nine milk production traits. Quadruplet cows in G+15781A were decided advantage in each milk production traits that 305-day milk yield, fat rate, protein rate and somatic cell scores (SCS) increased following with birth order. A allele and T allele had positive effect on SCS. In conclusion, this study showed that the haplotype AA may be a genetic marker on mastitis and other performance for Chinese Holstein cows.

Keywords Clusterin · Promoter · SNP · Haplotype · Milk production traits · Mastitis

Z. Wang · G. Wang (✉)
College of Animal Science and Technology, Nanjing Agricultural University, No. 1 Weigang, Nanjing 210095, Jiangsu, People's Republic of China
e-mail: glwang@njau.edu.cn

J. Huang (✉) · J. Zhong
Center of Dairy Cattle Science Research, Shandong Academy of Agricultural Sciences, Jinan 250100, People's Republic of China
e-mail: huangjinm@sina.com

Bovine mastitis is one of the most deleterious diseases inflammation of the mammary gland of dairy cattle and is usually a consequence of microbial infection and affected by genetic contagious [1]. It is a highly prevalent and costly disease for the dairy industry worldwide [2, 3]. Clusterin (CLU) was first described in 1983 as a major secretory glycoprotein produced by ram Sertoli cells [4]. It is normally secreted from mammalian cells and is both widely distributed and highly conserved. Between different mammals, about 70–80% of the amino acid sequence of Clusterin is identical [5]. One of the most striking things about CLU is the breadth of its biological distribution [6, 7]. In animal tissues CLU mRNA is near ubiquitous, including breast milk [8]. Furthermore, increased CLU expression is found in a variety of disease states [9], such as during mastitis of the dairy cow [10]. These implied that there was a possible relationship between the CLU and mastitis in dairy cows. So, it's of importance to examine the effect of CLU on mastitis for accelerating the breeding in Chinese Holstein cows.

Materials and methods

Animal, mammary, DNA and RNA collect

The 1,137 Chinese Holstein cows from nine dairy farms were used in this study and milk samples were taken from each cow once a month during routine milking in the whole lactation period. Data for milk production traits (305-day milk yield, fat rate, protein rate, fat protein ratio, fat yield, protein yield, milk yield and somatic cell score) were collected from the laboratory of the Dairy Herd Improvement Center (OX Biotechnology, Shandong, China). Genomic DNA from 1,137 cows was isolated from 3.8%

sodium citrate-treated blood samples using the phenol–chloroform method (with a 30 min centrifugation at 3000 rpm at 4°C) [11] and stored at −20°C. The content of DNA was estimated spectrophotometrically and diluted to the 50 ng μl^{-1}. Twelve mammary (infected and healthy) were collected aseptically base on the clinical symptoms and bacteriological test from 6 slaughtered Chinese Holstein cows and frozen in liquid nitrogen for RNA analysis. Total RNA was extracted by TRNzol Kit (TaKaRa) and concentration to produce cDNA (TaKaRa RNA PCR Kit Ver.3.0) immediately.

Cloning, sequencing and bioinformatics analysis

Primers P_T and P_B (Table 1) were designed to amplify the 5′UTR and the coding regions of the bovine *CLU* gene (GenBank accession NC_007306.4). PCR reactions were performed in 25-μl volumes and contained 100 ng genomic DNA, 2.5 μl 10× PCR Buffer, 1.8 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} dNTPs, 0.8 μmol l^{-1} of each primer and 0.5 U of Taq DNA polymerase (TaKaRa). The PCR program comprised an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 8 min, after end of reaction all PCR products were electrophoresed on 1% agarose gels. PCR products of P_T were purificated and connected pEASY-T3 vector were transformed into *E. coli* DH5α and selected positive plasmid to sequence for finding the 5′-upsteam region of Chinese Holstein cows and SNPs. PCR products of P_T were sequenced for the SNPs identification. Prediction of TATAbox using Hamming-Clustering Method for TATA Signal Prediction in Eukaryotic Genes (http://zeus2.itb.cnr.it/~webgene/wwwHC_tata.html), transcription factors prediction used WWW Signal Scan (http://www-bimas.cit.nih.gov/molbio/signal/) and TFSEARCH: Searching Transcription Factor Binding Sites (ver1.3) (http://www.cbrc.jp/research/db/TFSEARCH.html), CpG island predicted by MethPrimer-Design Primers for Methylation PCRs: (http://www.urogene.org/methprimer/index1.html) and CpG islands revealing Select parameters: (http://zeus2.itb.cnr.it/cgi-bin/wwwcpg.pl?page=ex).

Genotyping for SNP of 5′UTR region and the coding region

Single-stranded conformation polymorphism (SSCP) was performed to genotype variations within the amplified regions of 223 bp in protomer for C-994T by P_t (Table 1). PCR product, 6 μl, was mixed with 9 μl denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanol and 0.025% bromophenol blue), heated in water at 98°C for 10 min and immediately chilled on ice for 5 min. Denatured DNA was subjected to 10% polyacrylamide gel electrophoresis (29 acrylamide: 1 bisacrylamide) in 1× TBE buffer at a constant voltage of 120 V and temperature of 4°C, 12 h [12]. The gel was stained with 0.1% silver nitrate. PCR-restriction fragment length polymorphism (RFLP) was performed to genotype variations within the amplified regions of 260 bp in the seventh exon for G+15781A by P_b (Table 1). G+15781A hold a natural *Hin*fI endonuclease restriction site (G^ANTC) at 111 locus. PCR products, 1 μl, was mixed with 1 μl *Hin*fI, 2 μl 10× Buffer and 6 μl ddH_2O, digested at 37°C for 16 h [13]. The digested products were detected by electrophoresis in 10% polyacrylamide gel (29 acrylamide:1 bisacrylamide) in 1× TBE buffer at a constant voltage of 120 V and temperature of 4°C, 4 h and stained with 0.1% silver nitrate.

Statistical analysis

The genotypic and allelic frequencies, value of χ^2, polymorphism information content (PIC), effective number of alleles (Ne) and heterozygosities (H) were calculated. The association of single nucleotide polymorphisms (SNPs) or genotypes of the *CLU* gene with SCS and milk production

Table 1 Primers used for amplification and sequencing of the bovine *CLU* gene

Primers	Sequence 5′–3′	Product sizes (bp)	TM (°C)	Methods
P_T	F:GGCGGTACCCCCAATCCCTTCTTCTCCTC	2036	60.0	Cloning and sequence
	R:GAAGATCTCTCTGCGTAGGGAACTGGAA			
P_B	F: TGGAGAAGGAGGCAAGATGAAGACT	1369	58.9	Sequence
	R: ACGCCCGATACTTGCAAAAGCAACT			
P_t	F:CTTAGGGCTCAAACATCC	223	53.0	PCR–SSCP
	R: CTTCGCTCATCTCCACC			
P_b	F: CGTGACCCGGTTGCCATT	260	53.0	PCR–RFLP
	R: CCCAGGTGAACTGCTCGT			

TM melting temperature, *F* forward, *R* reverse, *SSCP* single-stranded conformation polymorphism analysis, *RFLP* restriction fragment length polymorphism

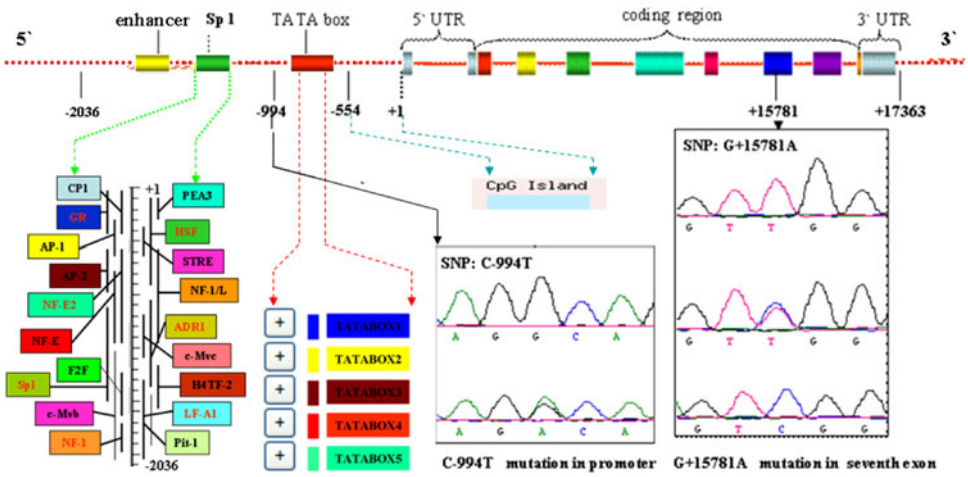

Fig. 1 Bioinformatics and SNPs of the *CLU* gene in Chinese Holstein cows

traits were analyzed by the least squares method as applied in SPSS software (13.0). The linkage disequilibrium analysis was performed by online SHEsis (http://analysis.bio-x.cn/myAnalysis.php) [14]. The substitution effect of gene was analyzed using the method described as Liu et al. [15]. The integrity of the animal model was analyzed using $Y_{ijkl} = \mu + h_i + p_j + s_k + m_l + e_{ijkl}$, where, Y_{ijkl} = observe value; μ = overall mean; h_i = the effect of genotype or combined haplotpe; p_j = effect of season and farm; s_k = effect of parity and birth order; m_l = effect of sire descent and e_i = random error. Value of $P < 0.05$ was regarded as significant. Multiple comparisons were performed by Duncan's method.

Results

Amplification, sequencing and bioinformatics analysis of the *CLU* gene in Chinese Holstein cows

The primer pairs PT to PB were used to amplify the 5′UTR region (2036 bp) and the mostly coding regions (1369 bp) of the bovine *CLU* gene, the transcription start site in exon 1 is shown by the first capital boldface G with +1, two novel SNPs(C-994T) before 5′UTR region and (G+15781A) in the seventh exon were sequenced and one enhancer was detected at −1828 bp site, five TATA box were predicted (−1084 bpAGTATTAATC-1094 bp, −1028 bpGGCATAAGGG-1038 bp, −552 bpTCCATTACAC-562 bp, −539 bpGTTATTTCTG-549 bp, −75 bpGCTATAAA TA-85 bp) nearby the transcription start site, one CpG island was found (Island size is 554 bp, GC Percent is 62%, Obs/Exp is 0.79) and One or more transcription factor binding sites were found in signal file of mammal of *CLU* gene (Fig. 1).

Genotype, allelic and genotypic frequency for SNPs of the bovine *CLU* gene in Chinese Holstein cows

Genotype by PCR–SSCP for C-994T(CC,CT) and PCR–RFLP for G+15781A(GG,GA,AA) (Fig. 2) and the allelic, genotypic frequencies, PIC, H, Ne, χ^2 values (Table 2) were significant differences and Chi-squared tests showed for SNPs did not meet with the Hardy–Weinberg equilibrium ($P < 0.05$) in Chinese Holstein cows.

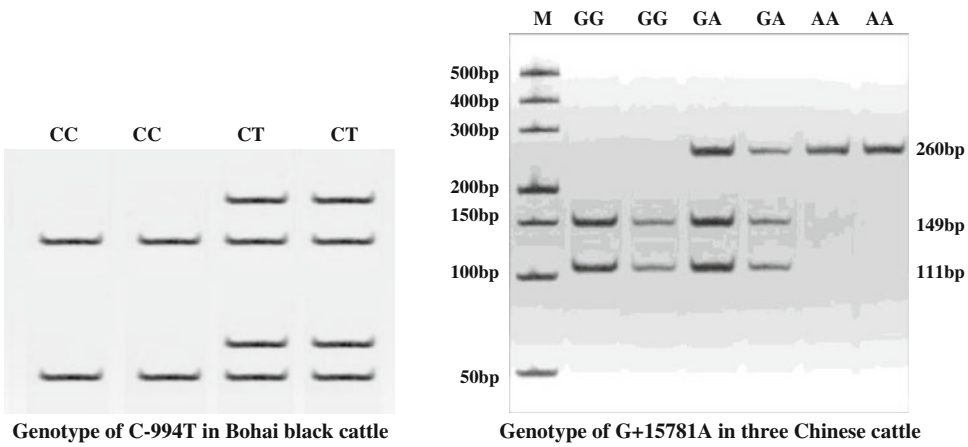

Fig. 2 Electrophoretic patterns of polymorphic loci in the *CLU* gene in three cattle breeds

Genotype of C-994T in Bohai black cattle

Genotype of G+15781A in three Chinese cattle

Table 2 Genotypic and allelic frequencies of SNPs in the *CLU* gene in three cattles

Loci	Genotypic frequencies			Allelic frequencies		PIC	H	Ne	χ^2
C-994T	CC	CT	TT	C	T	0.11	0.12	1.14	5.43
	0.87	0.13	0.00	0.94	0.06				
G+15781A	GG	GA	AA	G	A	0.34	0.44	1.78	6.79
	0.48	0.40	0.12	0.68	0.32				

Effect of the polymorphisms and alleles in the *CLU* gene on milk production traits in Chinese Holstein cows

For the *CLU* G+15781A polymorphism, the milk fat rate of AA genotype cows was higher than that of GG genotype cows, A allele at G+15781A tended to have increased SCS and it dropped in other milk production traits. But T allele at C-994T had positive effect on all milk production traits (Table 3).

Association of the combined haplotype of *CLU* gene with milk production traits in Chinese Holstein cows

The linkage disequilibrium analysis showed that two SNPs were not completely linked in Chinese Holstein cows ($D' = 0.494 < 0.075$, $r^2 = 0.007 < 0.33$) by SHEsis. Therefore, two SNPs were used for the haplotype analysis. A total of six combined haplotypes were found. The haplotype H1H4 (AGCT) significantly higher than H3H4 (GGCT) on fat and milk yield ($P < 0.05$), which decided advantage in nine milk production traits (Table 4).

Association of the *CLU* polymorphisms with milk production traits in Chinese Holstein cows of different purity

Each different *CLU* genotypes in G+15781A and C-994T showed significant differences with milk production traits except fat yield in different purity Chinese Holstein cows and 305-day milk yield, fat rate, protein rate and somatic cell scores (SCS) increased following with purity. Quadruplet cows in G+15781A were assured advantage in each milk production traits (Table 5).

Discussion

2036 bp fragments were obtained before 5′UTR of *CLU* gene and canonical promoter TATA box was found at the position between −75 and −85 bp, 554 bp CpG island (Criteria used: Island size >100, GC Percent >50.0, Obs/Exp >0.6) showed transcription start of smooth in low methylation. One SNP(C-994T) made the enhancer disappear and more transcription factor binding sites to move forward 80 bp at the promoter region, and reappear GATA-1(+451) and GT-I(−1918), which may alter regulation of gene expression [16, 17], sequentially, influence dysfunction or associate with disease [18, 19] and affect the ability of the protein to recognize its related ligands [20, 21], another SNP(G+15781A) transition was a synonymous mutation located at coding regions (1036 bp) in the seventh exon, which may affect the process of gene mRNA splicing or transcription [22, 23].

The allelic and genotypic frequencies, PIC, H, Ne and χ^2 values for SNPs showed considerable variability among Chinese Holstein cows, the allelic frequency of both the G and C alleles was up to 0.5, but the C allele with a lower heterozygosity in Chinese Holstein cows. PIC, H and Ne of SNP(C-994T) were lower than SNP(G+15781A), showed the lower polymorphism, lower genetic variability, lower ability to maintain allelic stability during selection or mutation, so SNP(C-994T) is unlikely to be useful in breeding programs for Chinese Holstein cows. Chi-squared tests up to 0.5 showed that SNPs did not meet with the Hardy–Weinberg equilibrium, but would be more expected to change the allelic and genotypic frequencies across next generation, it may be a greatly changed genetic structure in natural or artificial selection process of varying environmental or economic conditions in Chinese Holstein cows.

This study showed that A allele had a positive effect on SCS, but a negative effect on other milk production traits. In contrast, the T alleles had a positive effect on all milk production traits. haplotype AA had significantly lower SCS, maybe used as a tolerance haplotype for mastitis in Chinese Holstein cows. SHEsis analysis showed two SNPs had four haplotype of H1(AC), H2(AT), H3(GC), H4(GT), which get nine combined haplotype of H1H1(AACC), H1H2(AACT), H1H3(AGCC), H1H4(AGCT), H2H2 (AATT), H2H4(AGTT), H3H3(GGCC), H3H4(GGCT), H4H4(GGTT), but had only six combined genotypic of H1H1, H1H2, H1H3, H1H4, H3H3, H3H4 in this study animal DNA samples. H1H4 assured advantage in nine milk production traits. So, this study indicated genotype was not practical to increase all the milk production traits simultaneously, but combined haplotype or genotypic could, consequently, the combined haplotype associated effects may be more useful in evaluating milk production traits and selecting the optimum alleles in my study again

Table 3 Milk production traits for different *CLU* genotypes in Chinese Holstein cows

Loci	Genotype	305 Days milk yield (kg)	Fat rate (%)	Protein rate (%)	Fat protein ratio	Fat yield (kg)	Protein yield (kg)	Milk yield (kg)	SCS
C-994T	CC	6034.81 ± 69.06	3.93 ± 0.03	3.04 ± 0.02	1.31 ± 0.01	242.79 ± 5.07	201.52 ± 3.51	6699.23 ± 111.28	4.28 ± 0.06
	CT	5815.83 ± 161.26	4.01 ± 0.08	3.15 ± 0.04	1.28 ± 0.02	234.07 ± 11.87	195.35 ± 8.83	6405.02 ± 277.25	4.04 ± 0.16
	α1	−2060.90	−1.27	−0.96	−0.44	−82.88	−68.43	−2305.28	−1.49
G+15781A	GG	5921.20 ± 91.29	3.89 ± 0.04^{C}	3.04 ± 0.02	1.29 ± 0.01	233.10 ± 6.60	195.88 ± 4.44	6542.35 ± 144.25	4.29 ± 0.08
	AG	6104.11 ± 100.65	3.97 ± 0.04	3.06 ± 0.02	1.32 ± 0.02	254.10 ± 7.47	208.11 ± 5.38	6830.20 ± 166.76	4.22 ± 0.10
	AA	6024.11 ± 188.79	4.12 ± 0.11	3.09 ± 0.04	1.34 ± 0.03	235.29 ± 13.98	196.19 ± 9.96	6596.37 ± 312.20	4.22 ± 0.17
	α2	96.38	0.10	0.02	0.02	7.90	4.28	116.16	−0.05

The uppercase superscript letters (C) denote significant difference $P < 0.01$; a different superscript letter or values without any letter denotes no significant difference. α1 and α2 mean gene substitution effects

Table 4 Milk production traits for different *CLU* combined haplotype in Chinese Holstein cows

	Frequencies	305 days milk yield (kg)	Fat rate (%)	Protein rate (%)	Fat protein ratio	Fat yield (kg)	Protein yield (kg)	Milk yield (kg)	SCS
H1H1	126 (0.11)	6033.80 ± 185.43	4.20 ± 0.10	3.16 ± 0.04	1.33 ± 0.03	267.81 ± 15.96	330.84 ± 61.51	7151.24 ± 370.78	4.29 ± 0.17
H1H2	12 (0.01)	6875.21 ± 325.99	4.13 ± 0.28	3.17 ± 0.09	1.33 ± 0.12	277.08 ± 33.39	221.92 ± 21.11	7067.33 ± 725.67	4.79 ± 0.53
H1H3	426 (0.38)	6088.48 ± 109.64	3.86 ± 0.04	3.01 ± 0.02	1.31 ± 0.06	239.51 ± 7.03	302.56 ± 47.34	6404.35 ± 157.13	4.25 ± 0.09
H1H4	30 (0.03)	6778.22 ± 292.93	4.20 ± 0.18	3.22 ± 0.07	1.30 ± 0.06	309.88 ± 29.05	240.88 ± 23.33	7838.57 ± 652.16	4.73 ± 0.40
H3H3	459 (0.40)	5924.17 ± 98.45	3.95 ± 0.05	3.05 ± 0.02	1.31 ± 0.01	236.27 ± 7.78	420.80 ± 66.58	6350.65 ± 180.14	4.20 ± 0.10
H3H4	84 (0.07)	5599.02 ± 214.23	3.82 ± 0.11	3.06 ± 0.05	1.27 ± 0.04	229.20 ± 13.48^{d}	438.53 ± 136.39	6341.65 ± 267.57^{bd}	4.19 ± 0.19

The lowercase superscript letters (b, d) denote significant difference $P < 0.05$; a different superscript letter or values without any letter denotes no significant difference

SCS somatic cell score

Table 5 Milk production traits for different *CLU* genotypes in Chinese Holstein cows of different purity

Loci	Genotype	Purity	305 days milk yield (kg)	Fat rate (%)	Protein rate (%)	Fat protein ratio	Fat yield (kg)	Protein yield (kg)	Milk yield (kg)	SCS
C-994T	CC	1	5673.09 ± 118.19^{Bcd}	3.76 ± 0.06^{bcD}	2.93 ± 0.03^{BD}	1.30 ± 0.02	246.66 ± 10.53	205.77 ± 6.79	7022.96 ± 214.47^{d}	3.52 ± 0.10^{BCD}
		2	6319.61 ± 123.52	3.94 ± 0.05^{D}	3.11 ± 0.03^{C}	1.29 ± 0.02	244.78 ± 8.16	211.85 ± 5.86^{c}	6901.36 ± 179.41^{d}	4.01 ± 0.11^{CD}
		3	6074.50 ± 156.63	3.93 ± 0.07^{D}	2.99 ± 0.03^{D}	1.32 ± 0.02	227.64 ± 10.00	186.25 ± 6.99	6371.24 ± 234.31	4.83 ± 0.14^{D}
		4	6144.00 ± 169.25	4.25 ± 0.08	3.18 ± 0.31	1.35 ± 0.03	250.66 ± 11.99	193.81 ± 9.01	6137.58 ± 283.52	5.47 ± 0.13
	CT	1	5179.38 ± 338.33^{bd}	3.89 ± 0.16	3.11 ± 0.08	1.25 ± 0.04	246.20 ± 29.80	201.81 ± 20.36	6876.54 ± 635.72	3.40 ± 0.24^{CD}
		2	6282.86 ± 153.94	4.05 ± 0.11	3.10 ± 0.06	1.32 ± 0.04^{d}	251.26 ± 10.61	205.69 ± 10.45	7004.96 ± 339.07	3.53 ± 0.29^{CD}
		3	5789.53 ± 344.61	4.25 ± 0.20	3.17 ± 0.07	1.34 ± 0.06^{D}	209.44 ± 19.96	176.26 ± 14.05	5470.09 ± 426.89	4.87 ± 0.33
		4	6393.66 ± 477.05	3.86 ± 0.14	3.35 ± 0.08	1.16 ± 0.04	208.24 ± 27.84	188.45 ± 26.46	5510.86 ± 764.37	5.31 ± 0.37
G+15781A	GG	1	5495.61 ± 170.39^{bd}	3.61 ± 0.08^{BCD}	2.96 ± 0.05^{bD}	1.24 ± 0.02^{cd}	228.28 ± 13.19	201.56 ± 8.93	6845.54 ± 286.37^{d}	3.58 ± 0.13^{bCD}
		2	6203.88 ± 149.24	3.96 ± 0.07	3.09 ± 0.03	1.30 ± 0.02	239.53 ± 10.65	208.55 ± 6.81^{d}	6911.53 ± 221.00^{D}	4.05 ± 0.14^{cD}
		3	5876.97 ± 226.91	3.98 ± 0.08	2.99 ± 0.04^{d}	1.33 ± 0.02	221.12 ± 15.25	183.14 ± 10.81	6246.39 ± 355.75	4.54 ± 0.18^{D}
		4	6157.88 ± 202.41	4.10 ± 0.08	3.13 ± 0.03	1.34 ± 0.04	241.19 ± 14.90	178.11 ± 9.78	5741.82 ± 318.13	5.55 ± 0.16
	AG	1	5745.26 ± 163.70	3.90 ± 0.08^{d}	2.93 ± 0.47^{bD}	1.36 ± 0.03	262.32 ± 16.12	205.66 ± 10.07	6918.20 ± 307.48	3.39 ± 0.15^{CD}
		2	6375.05 ± 183.70	3.93 ± 0.07	3.11 ± 0.05^{d}	1.30 ± 0.03	261.90 ± 11.50	222.32 ± 9.85	7216.41 ± 288.97	3.78 ± 0.19^{CD}
		3	6324.54 ± 196.67	3.97 ± 1.01	3.03 ± 0.05^{d}	1.32 ± 0.03	236.26 ± 11.78	196.09 ± 8.12	6562.74 ± 277.17	4.90 ± 0.18^{d}
		4	6016.29 ± 317.99	4.18 ± 0.81	3.30 ± 0.03	1.27 ± 0.03	254.47 ± 21.02	210.24 ± 18.06	6426.35 ± 560.70	5.52 ± 0.19
	AA	1	5552.85 ± 341.22^{c}	3.94 ± 0.17^{d}	2.97 ± 0.07	1.32 ± 0.04	259.30 ± 32.01	216.39 ± 21.67^{d}	7802.68 ± 694.52^{C}	3.59 ± 0.20^{C}
		2	6538.73 ± 315.88^{C}	4.01 ± 0.12^{d}	3.15 ± 0.06	1.30 ± 0.05^{d}	229.05 ± 18.38	194.10 ± 13.83^{c}	6254.80 ± 408.05^{c}	3.95 ± 0.24
		3	4929.83 ± 439.59^{D}	4.05 ± 0.37^{d}	3.07 ± 0.08	1.32 ± 0.11	171.28 ± 30.40	117.78 ± 15.79^{D}	4097.22 ± 512.92^{d}	6.07 ± 0.58
		4	6783.33 ± 182.28	4.88 ± 0.43	3.21 ± 0.08	1.51 ± 0.12	242.44 ± 14.67	218.94 ± 15.15	6656.22 ± 303.39	4.61 ± 0.48

The same uppercase superscript letter (B, C, D) denotes significant difference $P < 0.01$; the same lowercase superscript letters (b, c, d) denote significant difference $P < 0.05$; a different superscript letter or values without any letter denotes no significant difference

[24], which same to conclusion of Fallin et al. [25] and Liu et al. [26].

Each genotype of SNPs in CLU showed significant differences with milk production traits except fat yield in different birth order Chinese Holstein cows, it main between triplet or quadruplet and first or second lactation. 305-day milk yield, fat rate, protein rate and somatic cell scores (SCS) increased following with birth order. This may be taken into consideration in breeding schemes designed to improve the Chinese Holstein cow population in terms of a higher 305-day milk yield, milk fat rate, milk protein rate. Such as quadruplet Chinese Holstein cows in haplotype AA was decided advantage in each milk production traits, but with the rise of parity, mastitis was increased (Via milk SCS).

In conclusion, our study firstly cloned and bioinformatics analysis the promoter of *CLU* gene in Chinese Holstein cows, two novel SNPs were revealed and investigated that the biological function of haplotype AA may be as genetic susceptibility factors for the progression of Chinese Holstein cow mastitis.

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