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Polymorphism in *PGLYRP-2* gene by PCR-RFLP and its association with somatic cell score and percentage of fat in Chinese Holstein

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ABSTRACT. Peptidoglycan recognition protein 2 (PGLYRP-2), which belongs to the *PGRP* family, is the only member that has no direct bactericidal activity but has N-acetylmuramoyl-l-alanine amidase activity. This feature of PGLYRP-2 indicates that it may play an important role in eliminating the pathogen associated molecular pattern (PAMP), such as peptidoglycan (PGN), which can reduce leukocytes in blood and lower somatic cell count (SCC) in milk. To investigate whether the *PGLYRP-2* gene is associated with mastitis and milk production traits in dairy cattle, the polymorphism of this gene was analyzed by PCR-RFLP in a population of 546 Chinese Holstein cows. A total of five single nucleotide polymorphism (SNP) loci were identified. The association analysis of a single SNP locus showed that the C+4867T locus was significantly associated ($P < 0.05$) with somatic cell score (SCS). Surprisingly, all loci were significantly associated ($P < 0.01$ or $P < 0.05$) with percentage of fat. Association analysis between combined genotypes and SCS and milk production traits indicated that H2H2 was associated with higher percentage of fat ($P < 0.05$). These findings demonstrated that SNPs in

PGLYRP-2 gene were related to mastitis resistance and percentage of fat, and that H2H2 would be a useful genetic marker of combined genotypes for breeding of Chinese Holstein.

Key words: *PGLYRP-2* gene; Mastitis; Percentage of fat; PCR-RFLP; PCR-PIRA; Polymorphism

INTRODUCTION

Mastitis, as one of the most widespread and severest diseases in dairy cattle, can lead to huge economic losses due to a reduction in production (Hortet and Seegers, 1998; Rajala-Schultz et al., 1999), treatment and culling. Mastitis is the inflammation of the mammary gland, which is mainly caused by pathogens from the environment or other cows suffering from contagious mastitis (Harmon, 1994). When pathogens invade the mammary gland, they are recognized by phagocytes such as mononuclear phagocytes and leukocytes through the special pathogen associated molecular pattern (PAMP). By presenting antigens and activating lymphocytes, the animal produces a large number of immune molecules and exerts an immunological effect to kill the pathogens. In the meantime, to control inflammation and avoid prolonged stimulation and septic shock (De Kimpe et al., 1995; Kengatharan et al., 1998), the animal must eliminate PAMP. There are two enzymes that can digest peptidoglycan (PGN) in mammals, lysozyme and N-acetylmuramoyl-l-alanine amidase. Lysozyme can hydrolyze the glycosidic bond between MurNAc and GlcNAc of PGN, while N-acetylmuramoyl-l-alanine amidase (NAMLAA, EC 3.5.1.28), primarily present in the serum, hydrolyzes the amide bond between MurNAc and L-Ala, which further reduces or eliminates the biologic activity of PGN. In recent years, some researchers have demonstrated that PGLYRP-2 is the N-acetylmuramoyl-l-alanine amidase exactly (Zhang et al., 2005). By knocking out the *PGLYRP-2* gene, investigators found that there was increased inflammation in *PGLYRP-2*$^{-/-}$ mice at 72 h post-infection, including significantly enhanced polymorphonuclear leukocyte accumulation and goblet cell depletion (Lee et al., 2012). It is believed that PGLYRP-2 plays a protective role during the late stage of inflammation, which protects the animal from an exaggerated inflammatory response. PGLYRP-2 can limit the overactivation of Th17 cells by promoting accumulation of Tregs at the site of inflammation (Park et al., 2011), since Th17 can produce large amounts of the pro-inflammatory cytokine IL-17, which can recruit neutrophils dramatically (Korn et al., 2009).

In dairy cows, when pathogens invade the mammary gland, there are plenty of leukocytes recruited to eliminate the pathogens, which is characterized by the increased number of somatic cell count (SCC) in the milk. In the meantime, to eliminate PAMP and prevent an exaggerated inflammatory response, PGLYRP-2 may play an important role in eliminating the PGN, which can reduce leukocytes in the blood and lower SCC in milk. Therefore, the SNP in the *PGLYRP-2* gene may have a relationship with SCC in milk.

MATERIAL AND METHODS

Animal and data collection

A total of 546 Chinese Holstein cattle with complete data of the 2nd-5th lactations

were randomly selected from the Xi'an Dairy Farm in China. SCC and milk production trait (305-day milk yield, percentage of fat and percentage of protein) data of the individuals sampled were collected from the Xi'an Dairy Cattle Center.

DNA preparation and PCR amplification

Genomic DNA was extracted from ACD-treated blood with the phenol-chloroform method (Sambrook and Russell, 2001) and stored at -80°C.

Seven pairs of primers P1-P7 (Table 1) were used to amplify the complete bovine *PGLYRP-2* gene. All primers were designed from a published genome sequence (GenBank accession number AC_000164.1). For screening the SNP locus in *PGLYRP-2* gene, the pooled DNA was used as template DNA in polymerase chain reaction (PCR). By sequencing the PCR product, the SNP site could be demonstrated in terms of overlapping peaks in chromatogram. SNP-RFLP analysis for the enzyme available was carried out by an online tool, WatCut. (http://watcut.uwaterloo.ca/watcut/watcut/template.php?act=snp_new). The PCR-primer introduced restriction analysis (PCR-PIRA) method was used to amplify the SNP sites to which no suitable enzyme could be applied directly (Jacobson and Moskovits, 1991). Primers, restriction enzymes and fragment sizes are shown in Table 1. The detection results of allelic variation were based on the electrophoretic pattern of the restriction enzyme-treated PCR products.

PCR was performed in a 10-µL reaction volume, containing 20-40 ng genomic DNA, 4 pmol each primer, 1X buffer (including 0.6 mmol $MgCl_2$), 80 µmol dNTPs and 0.6 U Taq DNA polymerase (Fermentas, Canada).

Table 1. Primer sets for PCR and PCR-primer introduced restriction analysis (PCR-PIRA) used in analysis of bovine *PGLYRP-2* gene.

Primers	Sequences of primers	AT[a] (°C)	SAF[b] (bp)	RE[c]
P1	F: 5'-CGTTGAGCCCATCCCACCTGA-3' R: 5'-CTGGAGTTTCCTAGAGACTTGAGCCT-3'	68.0	967	-
P2	F: 5'-GAAGCCAGGACTCTTAGTTCAGT-3' R: 5'-GAGACACAGACAAAAGGTTAAGGAGC-3'	65.5	964	-
P3	F: 5'-CAGCCACCTTCACTACTTACCTC-3' R: 5'-GTCCCAGCATCCCTCAGTTC-3'	65.5	1057	-
P4	F: 5'-GCCACCTCTCCAGAAGTTAGAC-3' R: 5'-CAAGTAAGGATCATCCGTCCAGT-3'	63.8	1004	-
P5	F: 5'-AGACTGATGTGAGAATGAACCGT-3' R: 5'-AGGTCTCGCTCCGCATC-3'	65.5	918	-
P6	F: 5'-GTATTTCTGGAGCGGCATTT-3' R: 5'-TGGACAGAGGAGCCTAGCAA-3'	61.5	905	-
P7	F: 5'-CCCAGCCACCCAACCTT-3' R: 5'-CAATAGTTGCAGCACTCTGGTTCG-3'	66.8	1006	-
T-609C	F: 5'-CGTTGAGCCCATCCCACCTGA-3' R: 5'-CTGGAGTTTCCTAGAGACTTGAGCCT-3'	68.0	967	EcoO109I
C-157T	F: 5'-AGACCCAGATGACCTTGGTGCA-3' R: 5'-TAGCCGGTTGACCTCAGTGGAG-3'	56.5	180	Alw44I
C+423T	F: 5'-AGTTATAGAGGAATTACTATGGTAAC-3' R: 5'-CGTGAAGAGGTTAAATTACC-3'	52.0	152	Eco91I
G+3359T	F: 5'-GGAGTCCAGACTTGGGAGAAGAACATGA-3' R: 5'-TCCAACTCCTCCGCAATTGGGACC-3'	61.5	112	EcoO109I
C+4867T	F: 5'-CTATCACCACCACCACCAGGATC-3' R: 5'-CAGGGAAGTCTCTCCAAGGTCTC-3'	58.5	165	BamHI

[a]AT = annealing temperature; [b]SAF = size of amplification fragment; [c]RE = restriction enzyme. The base with underline was mismatch, which aimed to adjust the melting temperature of primer. The base in box was mismatch to introduce an artificial restriction site into a PCR product for PCR-RFLP analysis.

The cycling protocol was 5 min at 95°C, 35 cycles of 94°C for 40 s, annealing temperature (Table 1) for 40 s and 72°C for 30-90 s (Table 1), and a final extension at 72°C for 10 min.

Data analysis

1. Genotypic and allelic frequencies were calculated using the POPGENE software (Version 1.32). The Hardy-Weinberg equilibrium of the mutations was determined by the χ^2 test.

2. Population genetic indexes, including gene heterozygosity (H_E), gene homozygosity (H_O), effective allele numbers (N_E) and polymorphism information content (PIC), were calculated according to Nei and Roychoudhury (1974). The formulas used were as follows: ("P_i" means the frequency of the ith allele, "n" means the number of alleles.)

$$H_o = \sum_{j=1}^{n} P_j^2 \quad \text{(Equation 1)}$$

$$H_E = 1 - \sum_{j=1}^{n} P_j^2 \quad \text{(Equation 2)}$$

$$N_E = 1 \Big/ \sum_{j=1}^{n} P_j^2 \quad \text{(Equation 3)}$$

$$PIC = 1 - \sum_{j=1}^{m} P_j^2 - \sum_{j=1}^{m-1} \sum_{j=j+1}^{m} 2P_j^2 P_j^2 \quad \text{(Equation 4)}$$

3. The pattern of pairwise linkage disequilibrium (LD) between the SNP loci was measured by LD coefficient (D') and correlation coefficient (r^2). Measurements and visualization of LD were determined using the HAPLOVIEW software (Version 4.2).

4. Haplotypes were reconstructed from population data using the PHASE program (Version 2.1).

5. The association analyses between genotypes of the single SNP locus and combined genotypes and somatic cell score (SCS) and milk production traits were performed by the least squares method which was applied in the general linear models (GLM) procedure of the SPSS software (Version 17.0). The following linear model was used to calculate within the least square means estimates (LSM) with standard errors for different genotypes and traits.

$$Y_{ijklm} = \mu + F_i + G_j + L_k + S_l + E_{ijklm} \quad \text{(Equation 5)}$$

Y_{ijklm} standed for the measured traits on each of the ijklmth animal, while μ for the overall population mean, F_i for fixed effect due to the i^{th} farm, G_j for the fixed effect of jth genotype/-combined haplotype, L_k for fixed effect due to the kth lactation number, S_l for fixed effect due to the lth calving season, and E_{ijklm} for the random error.

6. The data of SCC and milk production traits were collected monthly (ten times in

each lactation period) from the beginning of the 2nd lactation to the end of 5th lactation. The averages of monthly SCC and milk production traits of each individual were also calculated in the meantime. Because the distribution frequencies of SCC are usually inhomogeneous, the SCC data should be transformed to somatic cell score (SCS) in the valid application of parametric analysis using the following formula (Shook, 1982):

$$SCS = \log_2^{(SCC/100)} + 3 \quad \text{(Equation 6)}$$

The unit of SCC was cells/μL.

RESULTS

SNP loci identified in *PGLYRP-2* gene

The bovine *PGLYRP-2* gene is located on chromosome 7. The coding region consists of three exons and encodes a protein of 654 amino acids. In the present study, the complete sequence of *PGLYRP-2* gene was amplified with the primer pairs P1-P7. Five SNP loci were identified by DNA sequencing; the names and related information of these loci are shown in Table 1. The SNP locus was named according to the first base A of the initiation codon, which was designated as +1. Five SNP loci were all located in introns.

Analysis of polymorphism of the *PGLYRP-2* gene

Five SNP loci detected by PCR-RFLP are shown in Figure 1. The χ^2 test illustrated that the genotypic frequencies within the cattle population were all in agreement with Hardy-Weinberg equilibrium (Table 2) except the locus G+3359T. According to the PIC classification of (PIC value < 0.25, low polymorphism; 0.25 < PIC value < 0.5, intermediate polymorphism; and PIC value > 0.5, high polymorphism), all five SNP loci were intermediate polymorphic.

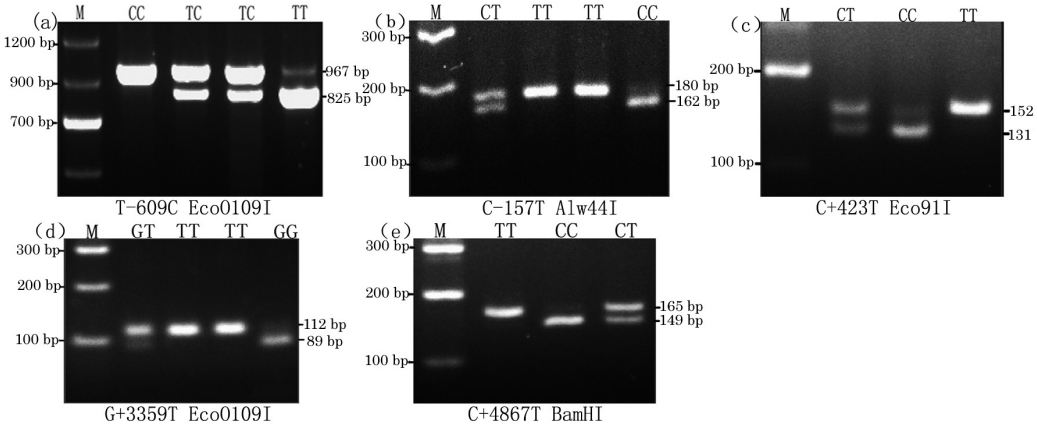

Figure 1. PCR-RFLP detection results of the PGLYRP-2 gene PCR product. Gel = 3% agarose gel electrophoretic patterns. *Lane M* = DNA molecular weight marker (marker I or marker II).

Table 2. Genotypic and allelic frequencies (%), value of χ^2 test, and diversity parameter of bovine *PGLYRP-2* gene.

Loci	Genotype (bp)	No.[a]	GF[b] (%)	Allele	AF[c] (%)	χ^{2d}	H_E[e]	H_O[f]	N_E[g]	PIC[h]
T-609C	TT: 825, 142	171	31.32	T	56.68	P > 0.05	0.5073	0.4927	1.9649	0.3705
	TC: 967, 825, 142	277	50.73	C	43.32					
	CC: 967	98	17.95							
C-157T	CC: 162, 18	143	26.19	C	51.37	P > 0.05	0.5037	0.4963	1.9985	0.3748
	CT: 180, 162, 18	275	50.37	T	48.63					
	TT: 180	128	23.44							
C+423T	CC: 131, 21	99	18.13	C	41.85	P > 0.05	0.4744	0.5256	1.9482	0.3683
	CT: 152, 131, 21	259	47.44	T	58.15					
	TT: 152	188	34.43							
G+3359T	GG: 89, 23	125	22.89	G	50.55	P < 0.05	0.5531	0.4469	1.9998	0.3750
	GT: 112, 89, 23	302	55.32	T	49.45					
	TT: 112	119	21.79							
C+4867T	CC: 149, 16	184	33.70	C	57.88	P > 0.05	0.4835	0.5165	1.9516	0.3687
	CT: 165, 149, 16	264	48.35	T	42.12					
	TT: 165	98	17.95							

[a]No. = genotype number; [b]GF = genotypic frequency; [c]AF = allelic frequency; [d]χ^2 = Hardy-Weinberg equilibrium χ^2 value; [e]H_E = gene heterozygosity; [f]H_O = gene homozygosity; [g]N_E = effective allele numbers; [h]PIC = polymorphism information content.

Association analysis of single SNP loci

The results of the association analyses between single markers and other experimental indices (SCS, 305-day milk yield, percentage of fat and percentage of protein) are shown in Table 3. Locus C+4867T was significantly associated with SCS ($P < 0.05$), whereas all SNP loci were significantly associated with percentage of fat ($P < 0.01$ or $P < 0.05$). The rest of the SNP loci had no significant association with SCS or milk production traits ($P > 0.05$).

Table 3. Associations analysis between 5 SNPs and SCS, milk production traits in Chinese Holstein.

SNP loci	Genotype	SCS	P value	305-day milk yield (kg)	P value	Fat rate (%)	P value	Protein rate (%)	P value
T-609C	TT	3.29 ± 0.14	0.161	25.86 ± 0.37	0.530	4.13 ± 0.05^A	0.003	3.06 ± 0.02	0.789
	TC	3.61 ± 0.09		26.34 ± 0.24		3.93 ± 0.03^B		3.04 ± 0.01	
	CC	3.53 ± 0.19		26.03 ± 0.49		3.95 ± 0.05^B		3.05 ± 0.03	
C-157T	CC	3.30 ± 0.16	0.274	25.40 ± 0.38	0.072	4.15 ± 0.06^A	0.002	3.06 ± 0.22	0.643
	CT	3.57 ± 0.09		26.34 ± 0.27		3.95 ± 0.03^B		3.04 ± 0.18	
	TT	3.54 ± 0.15		26.51 ± 0.38		3.93 ± 0.05^B		3.05 ± 0.24	
C+423T	CC	3.26 ± 0.17	0.298	25.41 ± 0.50	0.172	4.15 ± 0.06^a	0.018	3.07 ± 0.23	0.364
	CT	3.55 ± 0.10		26.40 ± 0.26		3.98 ± 0.04^b		3.04 ± 0.19	
	TT	3.54 ± 0.13		26.16 ± 0.33		3.94 ± 0.04^b		3.06 ± 0.22	
G+3359T	GG	3.39 ± 0.17	0.709	25.41 ± 0.41	0.122	4.10 ± 0.06^a	0.044	3.07 ± 0.21	0.539
	GT	3.53 ± 0.09		26.39 ± 0.25		3.98 ± 0.03^{ab}		3.04 ± 0.19	
	TT	3.52 ± 0.16		26.22 ± 0.42		3.91 ± 0.05^b		3.04 ± 0.25	
C+4867T	CC	3.49 ± 0.13^a	0.031	26.41 ± 0.33	0.313	3.95 ± 0.04^b	0.018	3.06 ± 0.22	0.399
	CT	3.64 ± 0.10^a		26.16 ± 0.26		3.97 ± 0.04^b		3.04 ± 0.19	
	TT	3.11 ± 0.16^b		25.55 ± 0.52		4.15 ± 0.07^a		3.07 ± 0.23	

Values with different letters within the SNP locus differ significantly at $P < 0.05$ (a, b, c) or $P < 0.01$ (A, B).

Linkage disequilibrium and haplotype analysis

LD between the five loci in the population was estimated (Table 4). The analysis showed that all loci were closely linked because there was strong LD ($|D'| > 0.7$ and $r^2 > 1/3$) between them.

Based on the results of Hardy-Weinberg equilibrium and LD analysis, the haplotype analysis with four loci showed that there were 16 different haplotypes and that four of the haplotype frequencies were greater than 5.00% (Table 5), which accounted for 88.89% of entire haplotypes.

Table 4. Estimated values of linkage equilibrium analysis between 5 SNP loci within PGLYRP-2 gene.

SNP loci	T-609C	C-157T	C+423T	G+3359T	C+4867T
T-609C	-	D' = 0832	D' = 0.915	D' = 0.932	D' = 0.910
C-157T	r^2 = 0.599	-	D' = 0.770	D' = 0.793	D' = 0.767
C+423T	r^2 = 0.461	r^2 = 0.404	-	D' = 0.864	D' = 0.935
G+3359T	r^2 = 0.678	r^2 = 0.608	r^2 = 0.526	-	D' = 0.855
C+4867T	r^2 = 0.461	r^2 = 0.405	r^2 = 0.864	r^2 = 0.521	-

Table 5. Haplotype and haplotype frequency within population studied of 4 SNPs in bovine *PGLYRP-2* gene.

Haplotype	SNP loci				Frequency in population	Cumulative frequency
	T-609C	C-157T	C+423T	C+4867T		
H1	C	T	T	C	0.3809	0.3809
H2	T	C	C	T	0.3555	0.7364
H3	T	C	T	C	0.1002	0.8366
H4	T	T	T	C	0.0523	0.8889

Association analysis of combined genotypes

The association analyses of combined genotypes were performed, and the results are shown in Table 6. There were five types of main genotype combinations which had a sample size of more than five individuals. The analysis suggested that there was a significant association between combined genotype H2H2 and higher percentage of fat. Although H2H2 had an obviously lower SCS, no significant association was found between them. As in the result for single locus analysis, combined genotype also had no significant association with 305-day milk yield and percentage of protein.

Table 6. Associations analysis between combined genotype and SCS, milk production traits in Chinese Holstein.

Genotype combination	No. of combination	SCS	305-day milk yield (kg)	Fat rate (%)	Protein rate (%)
H1H1	69	3.39 ± 0.23	26.18 ± 0.58	3.97 ± 0.06^{b}	3.07 ± 0.03
H1H3	47	3.47 ± 0.23	26.36 ± 0.64	3.97 ± 0.08^{b}	3.08 ± 0.02
H1H4	23	3.52 ± 0.30	26.94 ± 0.51	3.83 ± 0.09^{b}	3.04 ± 0.04
H2H2	68	2.99 ± 0.19	25.19 ± 0.58	4.22 ± 0.08^{a}	3.07 ± 0.03
H3H4	6	3.82 ± 0.92	28.85 ± 0.97	4.02 ± 0.18^{ab}	3.03 ± 0.05
P-value	-	0.416	0.195	0.018	0.965

Values with different letters within the same column differ significantly at $P < 0.05$ (a, b, c).

DISCUSSION

PGRP is an important pattern recognition molecule (PRM) of the innate immune system (Tydell et al., 2006). In mammals, it is a protein family with four members: PGLYRP-1, PGLYRP-2, PGLYRP-3 and PGLYRP-4, which were originally named as PGRP-S, PGRP-L, PGRP-Iα and PGRP-Iβ, respectively, standing for short, long and two intermediate transcripts,

respectively (Dziarski and Gupta, 2006). Interestingly, although PGLYRP-2 belongs to this *PGRP* family, it is the only one that has no bactericidal activity but has N-acetylmuramoyl-l-alanine amidase activity. This specific feature of PGLYRP-2 may decide its special role in innate immunity which differs from the others. In mammals, PGLYRP-2 and lysozyme can hydrolyze PGN to the less or non-bioactive fragment synergistically (Wang et al., 2003). It is very important for the organism to avoid exaggerated and prolonged inflammation response. Research has demonstrated that PGLYRP-2 has other roles in immunity besides hydrolyzing PGN. Sang et al. (2005) reported that silencing of *PGLYRP-2* gene in intestinal cells challenged with *Listeria monocytogenes* results in down regulation of β-defensin-1. Conversely, overexpression of *PGLYRP-2* gene can dramatically upregulate the expression of β-defensin-1 in porcine cells (Sang et al., 2005). Additionally, peritoneal macrophages from *PGLYRP-2*-deficient mice produce less interleukin-6 and tumor necrosis factor alpha when stimulated with *Escherichia coli* or lipopolysaccharide (Xu et al., 2004). These reports indicated that PGLYRP-2 had an important role in regulating the immune molecule.

In all single SNP loci, only locus C+4867T was associated with SCS. To date, there is no research indicating that the *PGLYRP-2* gene is connected with fat synthesis during milk production. Surprisingly, our work showed that all SNP loci were significantly associated with percentage of fat. In mammals, PGLYRP-2 acts as an amidase and plays an essential role in hydrolyzing PGN. So far, some researchers have found that the *PGLYRP-2* gene has a different alternative splice in the mouse (Kibardin et al., 2003), pig (Sang et al., 2005) and human (UniProtKB/swiss-prot, isoform: Q96PD5-1 and Q96PD5-2), which means that the *PGLYRP-2* gene in cattle may also have alternative splicing. Alternative splicing may change the open reading frame (ORF) and lead to a protein with a function affecting percentage of fat. On the other hand, PGLYRP-2 has two predicted transmembrance domains by bioinformatics analysis (Liu et al., 2001), which may imply that PGLYRP-2 has a special role in transporting specific materials.

After the analysis of combined genotype, H2H2 showed a significant association with higher percentage of fat and an association with lower SCS, though the difference did not reach the level of significance. According to the results, H2H2 can be considered a candidate gene in molecular marker-assisted breeding.

In conclusion, five SNP loci in an intron of the *PGLYRP-2* gene were identified in this study. We found that locus E was associated with SCS, and all the loci were associated with percentage of fat. Association analysis between combined genotypes and SCS and milk production traits indicated that H2H2 was associated with higher percentage of fat. To sum up, SNPs in *PGLYRP-2* gene were associated with SCS and percentage of fat in Chinese Holstein. H2H2 could be a useful genetic marker of combined genotypes for Chinese Holstein breeding. Further studies of PGLYRP-2 functional mechanisms and bioactivity, such as validation of the various allelic effects on SCS and percentage of fat, are still needed to be analyzed in a larger population.

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