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SNPs in splicing region and miRNA binding region of *Bos taurus* TREM-1 gene reveals its association with mastitis

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ABSTRACT

Proper splicing is important for the functioning of a gene, and any interruption in splicing causes several deleterious events. Triggering receptors present on myeloid cells, TREM-1, are implicated in inflammation and act as amplifiers by mediating the release of proinflammatory chemokines and cytokines in response to fungal and bacterial infections. In bovines, mastitis is an inflammatory disease in which mammary gland inflammation is generally caused by bacteria. We found rs109937179 and rs208224995 SNPs in the splicing and miRNA binding region of TREM-1 gene in Chinese Holstein cows. The genotype distribution of the alleles for TREM-1 (rs109937179 and rs208224995) gene polymorphisms was investigated in 364 and 320 Chinese Holstein cows, respectively. We found that the GG genotype of the rs109937179 polymorphism and rs208224995 genotype of CA within the TREM-1 gene were associated with an increased risk of mastitis. Importantly, rs109937179 was found in the splicing region of TREM-1, and rs208224995 has a miRNA binding region for bta-miR- 2329-3p in the 3'UTR, which determines its effective roles in gene expression regulation.

Keywords: Chinese Holstein, miRNA binding region, Splicing region

INTRODUCTION

Genetic polymorphisms are playing an increasingly important role as DNA markers in designing animal breeding plans. Single nucleotide polymorphism (SNP) based genomic selection is one of the powerful tools in livestock marker-based selection (Seidel, 2010). SNPs result in wide functional consequences because of their location on the gene where it has the potential to bring changes to protein including splicing region implications, at start and or stop codons, and at miRNA binding regions (Gurgul *et al.*, 2019). A more stringent breeding plan can be designed if such information is known about the SNPs.

A healthy immune system is key to the high quality and quantity of milk in dairy cows. Mastitis remains a major concern to the dairy industry and a robust breeding plan that can assure improvements against prevailing concerns of mastitis is highly encouraged (Farmanullah *et al.*, 2021). The intramammary infection that includes 75% of leukocytes and 25% of epithelial cells is determined as somatic cell count and is considered a good predictor of mastitis (Sharma *et al.*, 2011). The genetic evaluation of mastitis is carried out by an algorithm where somatic cell count is transformed into somatic cell score to maintain distribution normality (Alam *et al.* 2015). Several research groups investigate the host genome for suitable targets that can bring resistance against mastitis especially the SNPs in immune-related genes (Bhattarai *et al.* 2017, Farmanullah *et al.*, 2021). TREM-1, or Triggering Receptor Expressed on Myeloid Cells 1, a cell surface receptor protein primarily found on neutrophils and monocytes (myeloid cells), plays an important role in the regulation of the immune response, particularly in the context of inflammation and innate immunity (Pandupuspitasari *et al.*, 2016). Several studies have demonstrated the association of TREM1 with inflammation and showed it as an important target gene for selection in inflammatory diseases (Bosco *et al.*, 2016, Verstockt *et al.*, 2019). Because of the important roles of TREM1. we decided to look for SNPs in TREM1 of Chinese Holstein and importantly the SNPs selected were found to be in the splicing region and miRNA binding region of TREM1.

MATERIALS AND METHODS

Ethical Statement

The experiments of this study were conducted according to the regulations approved for animal experiments by Ministry of Science and Technology China in 2004, and also approved by Animal Care and Use Committee from the Dairy Cattle Research Center, Hubei Academy of Agricultural Sciences, Hubei, P. R. China.

Dairy Cows Management and Blood Sampling

Chinese Holstein cows from a farm located in the sub-tropical region of Hubei province in China with an average temperature of 16-17 C, 1100-1200 mm annual rainfall and 75% relative humidity. Dairy cows, fed with a total mixed ration thrice daily, housed in free stall barns, were employed to collect 10 ml blood samples in sterile EDTA tube containing anticoagulants. The blood samples were stored at -20 C before extracting genomic DNA. DNA was extracted as described previously by (Wang *et al.*, 2011). The extracted DNA was quantified using NanoDropTM ND-2000c Spectrophotometer (Thermo Scientific, Inc.).

Primer Design, Sequencing and Genotyping

TREM1 gene sequence of *Bos taurus* was retrieved from NCBI and five pairs of primers were made using Primer Premier 5·0 software and synthesized by Sangon Biological Engineering Technology (Shanghai, China) shown in table 1. Briefly, a PCR mixture of 20 µl was prepared containing 2 µL 10 X buffer, 0.6 µL 10 mM dNTPs, 0.4 µL 10 µM of each primer, 2.0 µL 50 ng/µL genomic DNA, 0.3 µL 5 U/µL Taq DNA polymerase (TaKaRa, China), and 14.3µL ddH2O. The PCR cycling protocol was set for 10 min at 94 °C, denaturing for 36 cycles at 94 °C for 45 seconds, annealing at 72 °C Tm°C for 45 seconds, final extending at 72 °C for 25 minutes and cooling at 15 °C for 15 minutes.

The PCR product was evaluated by running on 2% agarose gel for 45 minutes, and was sequenced in both directions by ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) using standard protocol. The restriction enzymes for genotyping were selected using http://watcut.uwaterloo.ca/template.php. The PCR products were digested with selected endonucleases for 8 hours at 37 °C. The digested PCR product stained with ethidium bromide was run on 2.5% agarose gel using gel electrophoresis and seen under UV light to determine the genotype of the animal.

Table 1. Primer and restriction enzymes used in the study

Gene	SNP	Sequence of Primers	Restriction enzymes	Annealing Temperature
TREM-1	rs109937179	F: GCCAAGTTCCTCCATTCATTCGTGT 25bp R: TCTTCCCCTTACACCCCTCTTCAGG 25 bp	AluI	61°C
TREM-1	rs208224995	F: GTGGACTCCTGAGTAAGACCCT 22bp R: TGTAGCCCAGGGTGAGGA 18bp	AluI	58°C

Table 2. rs109937179 and rs208224995 genotyping and allele frequencies

Gene	SNP locus	SNP ID	Type	Enzyme	Genotype	Length of fragment (bp)	Genotypic frequencies	Allele frequencies	Equilibrium
TREM-1	23:15148623 (G/A)	rs109937179	Splice variant	AluI	GG	691	320(87.91%)	G(0.89) A(0.11)	Disequilibrium (36.67)
					GA	187/504/691	10(2.74%)		
					AA	187/504	34(9.34%)		
	23:15148422 (C/A)	rs208224995	3'UTR	AluI	CC	175/454	163(5.93%)	C(0.6) A(0.4)	Disequilibrium (30.06)
					CA	175/454/629	58(18.93%)		
					AA	629	99(30.12%)		

Table 3. Association study of rs109937179 and rs208224995

Gene	SNP ID	Genotype	Genotype frequency	SCS	SCC (x1000/ml)	Milk Fat (%)	Milk Protein (%)	305 Days Milk (kg)
TREM-1	rs109937179	GG	320	4.03 ± 0.03^{b}	203.66 ± 7.34^{b}	3.71±0.03	3.12±0.02	7740.25 ± 89.10^{a}
		GA	10	3.95 ± 0.03^{ab}	193 ± 6.27^{ab}	3.75±0.03	3.13±0.02	7334.00 ± 143.84^{ab}
		AA	34	3.94 ± 0.02^{a}	192 ± 6.19^{a}	3.72±0.02	3.14±0.03	7824.68 ± 288.78^{b}
	rs208224995	CC	163	3.99 ± 0.02^{ab}	199 ± 6.13^{ab}	3.73 ± 0.03^{ab}	3.15±0.02	7560 ± 136.52^{ab}
		CA	58	4.03 ± 0.08^{b}	204 ± 6.85^{b}	3.70 ± 0.02^{a}	3.10±0.05	7491 ± 336.03^{a}
		AA	99	9.96 ± 0.04^{a}	195 ± 4.13^{a}	3.84 ± 0.08^{b}	3.15±0.03	8644 ± 203.54^{b}

Different superscripts in the same column indicate a significant different at $P<0.05$.

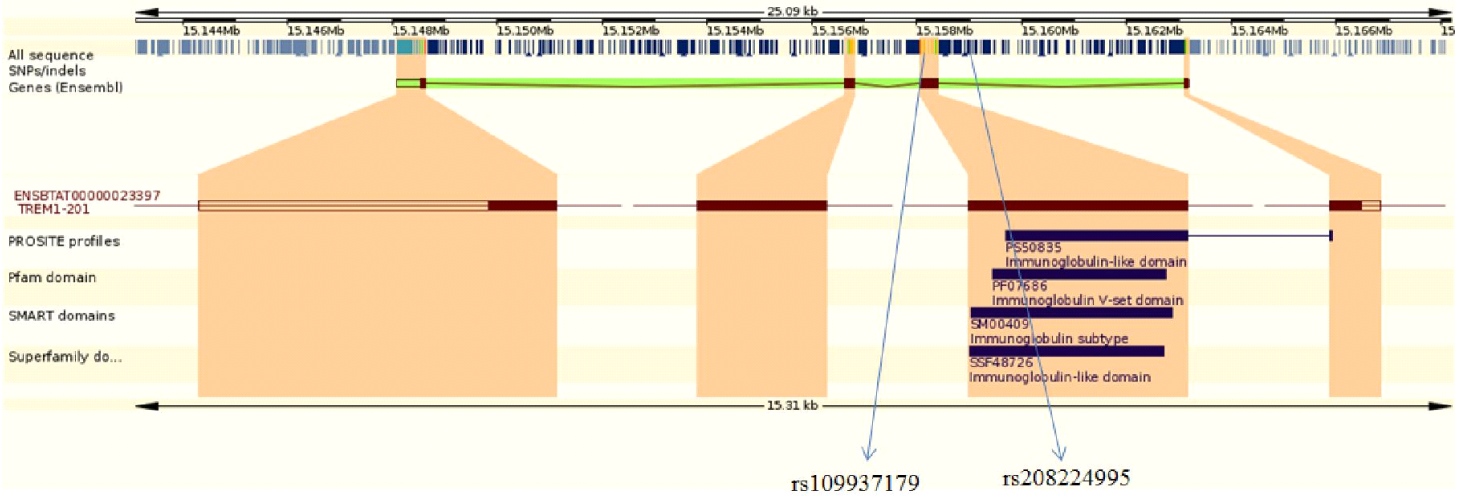

Figure 1. SNPs including two rs109937179 and rs208224995 in TREM-1 gene.

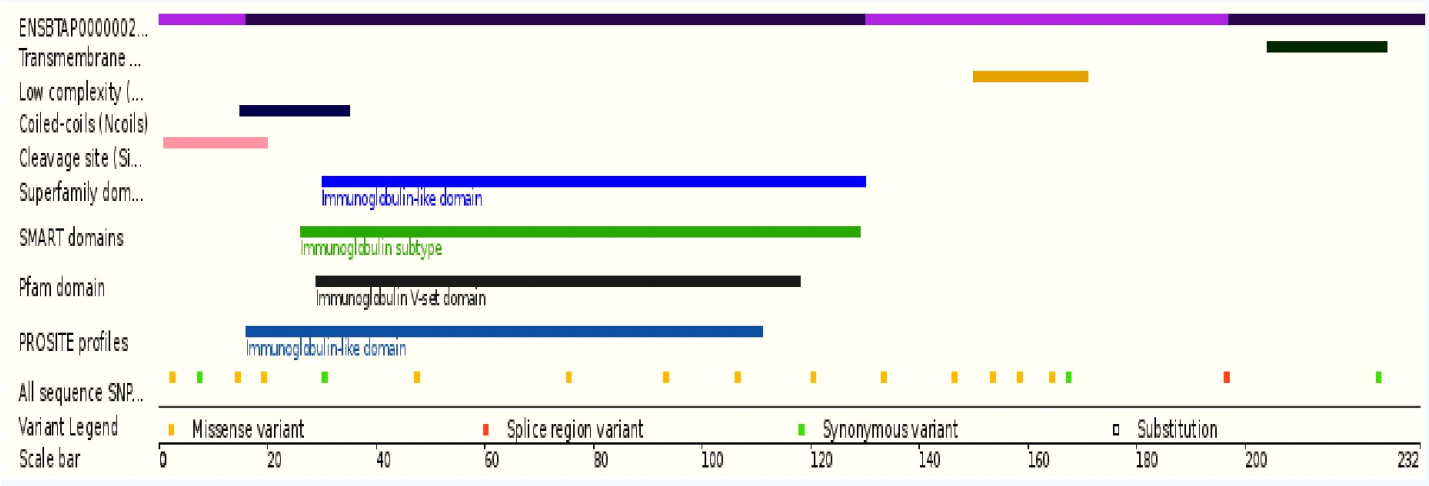

Figure 2. SNPs in exonic region of TREM-1 gene

Statistical Analysis

The Hardy-Weinberg Equilibrium test was conducted keeping P value ≤ 0·05 and SNPs that deviated from Hardy-Weinberg equilibrium were filtered. The association analysis for the retained SNPs and traits (305d milk yield: milk fat percentage (%), milk protein percentage (%), somatic cell count SCC and somatic cell score SCS). Frequency of each gene is calculated according to Nei (1987):

$$2nij - \sum n2\ qi = ij$$

2N Where: Qi = frequency of gene-i, nij=number of individuals to genotype, AiAj nii is number of individuals to genotype and AiAi N is number of sample.

Frequencies of distribution of alleles to know Hardy Weinberg Equilibrium within the herds was compared using the Chi-square test.

$$\chi 2 = \sum \frac{n\ (Q_i - E_i)2}{i\ i}$$

i =1 Ei Where:

X2 = Chi-square test, Qi = number of individual given phenotype observed, Ei = number of that phenotype expected from null hypothesis were analyzed using general linear model (GLM) Exact test of Hardy–Weinberg Equilibrium was conducted before the association study. SNPs were filtered for deviation from Hardy-Weinberg Equilibrium (P-value ≤ 0·05). The associations between the retained SNPs and the traits (fat percentage, protein percent- age, SCS, 305 d milk yield) were analysed using the general linear model (GLM) procedure of SAS 9.13. The general linear model is as follows:

Yijk =μ+ hi + Pj + Qk + Nl + eijkl

Where Yijk = the analyzed trait of cow, μ is general mean, hi is farm effect, Pj is year and season effect, Qk is parity effect, Nl is genotype effect and eijkl is random error effect.

RESULTS

SNPs in TREM-1

We analyzed single nucleotide polymorphisms (SNPs)) in the TREM-1 gene of Bos taurus from www.ensembl.org. We found that there were 13 missense variants, 3 splice region variants, 5 synonymous variants. 42 3'UTR variants, 544 intron variants, 136 upstream gene variants, and 272 downstream gene variants. The SNPs in TREM1 gene of *Bos taurus* are given in figure 1. We also analyzed the SNPs in the exonic region of TREM1 gene of *Bos taurus* using Ensembl database and found that there are 13 missense variants, 3 splice region variants, and 5 synonymous variants shown in Figure 2.

rs109937179 and rs208224995 Genotyping of TREM-1

PCR-RFLP method was used to identify the genotypes of TREM1 gene. The following DNA restriction fragments were obtained for the TREM-1 SNP rs109937179, 691bp (GG genotype), 187/504/691bp (GA genotype) and 187/504bp (AA genotype) and rs208224995 polymorphism: 175/454bp (CC genotype), 175/454/629bp (CA genotype), and 629bp (AA genotype) as shown in Figure 3).

Genotype Frequencies and Allele Frequencies of rs109937179 and rs208224995

The rs109937179 frequency for allele G (0.89) is higher than allele A (0.11). It was found that the GG genotype was the most frequent in the dairy cows population (87.912%), followed by AA (9.341%), whereas the GA was the least frequent (2.747%). The rs208224995 frequency for allele C (0.6) is higher than allele A (0.4). The CC genotype was found the most frequent in the population (50.938%), followed by AA (30.125%), whereas the CA was the least frequent (18.938%). The population was in disequilibrium at these sites after analyzing for Hardy-Weinberg equilibrium as shown in Table 2. Moreover, the SNPs rs109937179 and rs208224995 show an association with SCC and SCS that corresponds to immunity as shown in Table 3. Table 3 shows that there is a significant association of both SNP sites with immunity traits (SCS and SCC). Genotype GG allele is higher than GA and AA alleles of rs109937179, it indicates genotype GG is highly associated with SCS and SCC. While for SNP

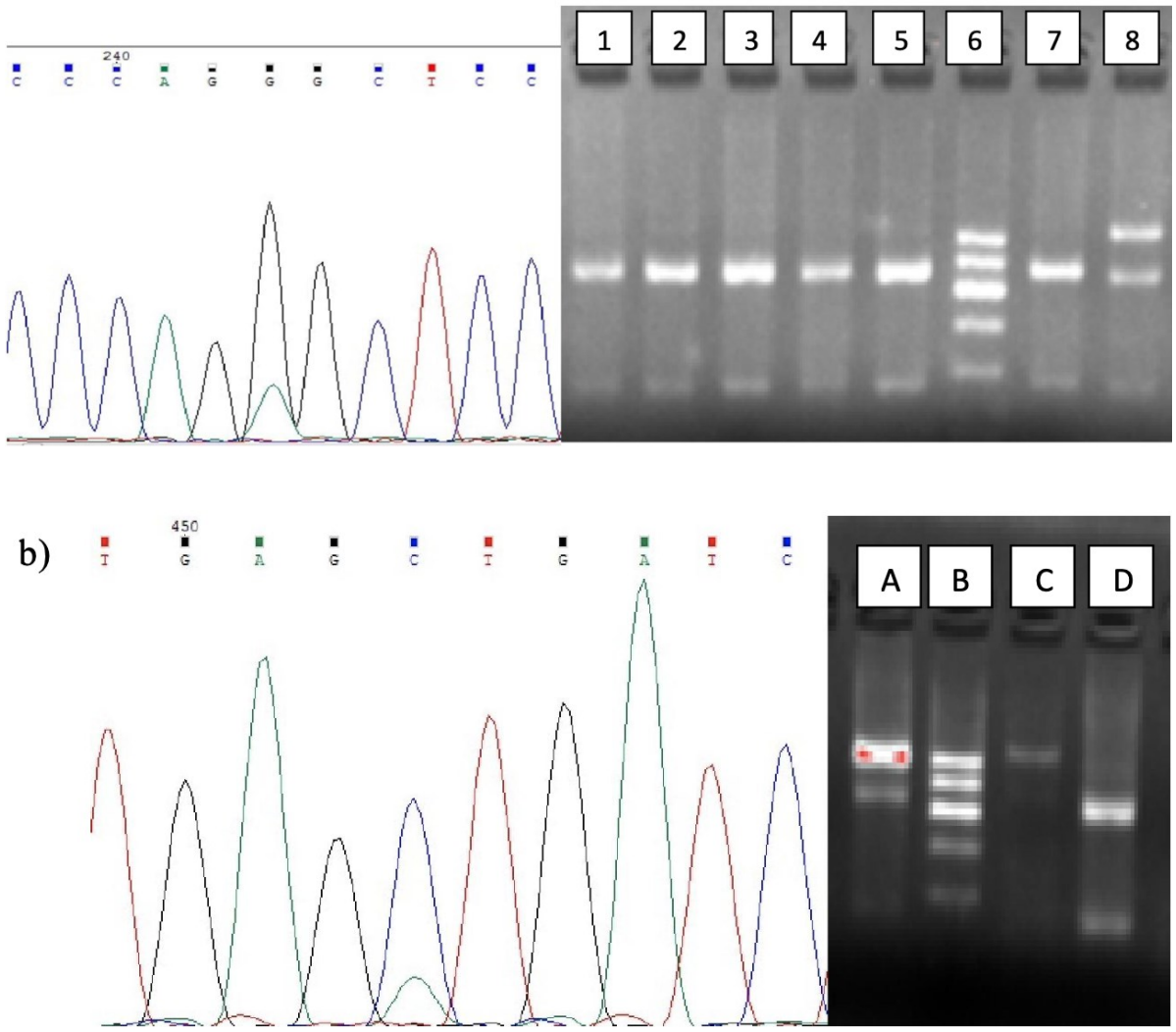

Figure 3. PCR-RFLP pattern of TREM-1 gene digested with AluI restriction enzyme. a) rs109937179: 1-5 and 7 is AA and 8 is GA b) rs208224995: A is CA, C is AA, and D is CC. B is DNA Marker I (600bp).

rs109937179, genotype AA has the highest 305 DM yield than alleles GA and GG, which indicated allele AA is highly associated with 305 DM yield.

In rs208224995, genotype CA allele is higher than AA and CC alleles which indicates allele CA is highly associated with SCS and SCC. While in the same rs208224995 allele AA is higher than alleles CC and CA, it shows that AA is highly associated with milk fat and 305 DM yield.

Predicted Location Characteristic of rs109937179 and rs208224995, the miRNA Binding Site and Splicing Region

The two SNPs rs109937179 and rs208224995 genotyped in dairy cows are predicted to be in the two important sites that is in the splicing region and miRNA binding domain which can regulate gene expression and produce splice variants. It is shown in Figure 4. Stemloop structure of miRNA is shown in Figure 5.

DISCUSSION

The triggering receptor expressed on myeloid cells 1 (TREM1) belongs to the family of Immunoglobulin (Ig) cell surface receptors and is expressed selectively by blood PMN, monocytes and macrophages and lacks signaling motif of the

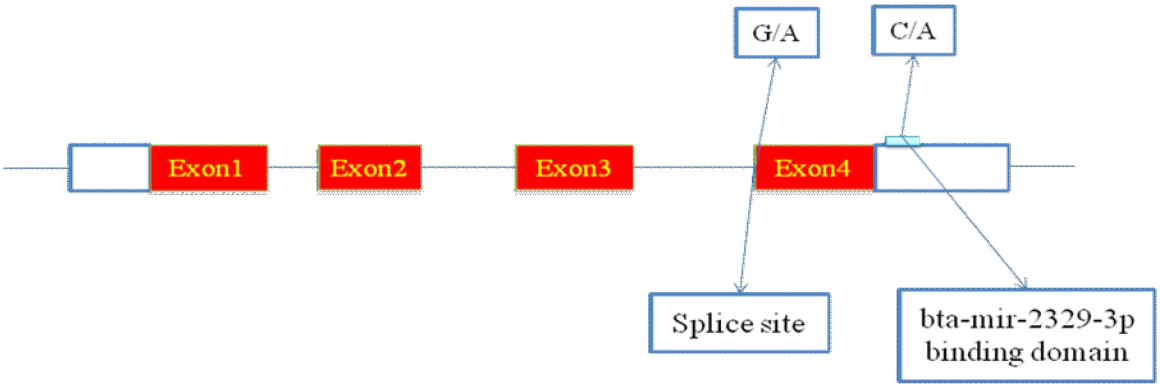

Figure 4. miRNA and splicing region prediction using miRbase

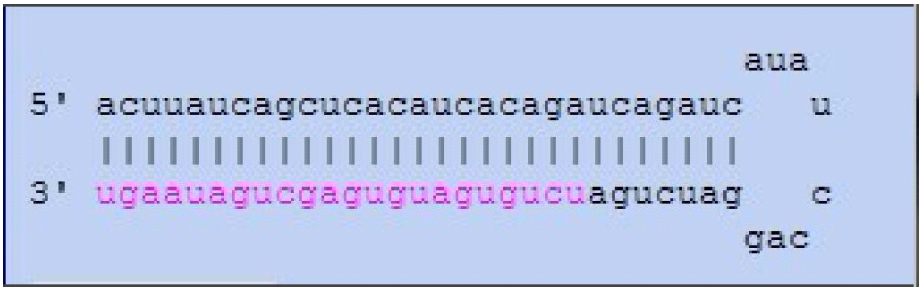

Figure 5. Stemloop bta-miR2329-3p sequence. The mature sequence of bta-miR-2329-3p: UCUGUGAUGUG-AGCUGAUAAGU

cytoplasmic domain thus its activation is carried out by DNAX activating protein DAP12 resulting in proinflammatory immune response (Moyes *et al.*, 2009). The high expression of TREM1 is associated with inflammatory diseases cardiovascular diseases and its downregulation is associated with 60% reduction of atherosclerosis, and it was demonstrated that SNP on TREM1 ie minor allele T of rs2234246 was associated with higher levels of serum TREM1.

Soluble TREM1 expression has been implicated in idiopathic granulomatous mastitis. It has been proposed that the inhibition of soluble TREM1 may have a significant role in controlling idiopathic granulomatous mastitis (Ates *et al.*, 2022). TREM1 gene was compared in inoculated low somatic scores ewes to control and it was found that TREM1 was among the top differentially expressed genes (Sophie, 2017).

Mastitis is an inflammatory condition of the mammary gland, which is generally caused by bacteria (Jaisue *et al.*, 2022). Triggering receptors expressed on myeloid cells (TREMs) are recognized as key players in the innate immune system that play a significant role in the recognition of infectious agents, particularly, bacteria (Pandupuspitasari *et al.*, 2016). In present study it was hypothesized that the single nucleotide polymorphism variations in TREM-1 gene may affect individual susceptibility to mastitis. The genotype distribution of alleles for TREM-1 (rs109937179 and rs208224995) gene polymorphisms was investigated in 364 and 320 Chinese Holstein cows respectively. We found that GG genotype of the rs109937179 polymorphism and rs208224995 genotype CA within TREM-1 gene was associated with an increased risk of mastitis.

Importantly, if the SNPs are found in gene

regulatory regions such as in miRNA binding site or splicing region can be of high importance. The SNP rs208224995 is predicted to have miRNA binding site which makes it very important as it can alter the expression of TREM-1. The rs109937179 is in the splicing region that has association with SCS and SCC, and thus can be seen as important SNP in regard of TREM-1 function.

CONCLUSION

We found that GG allele is significantly higher than alleles GA and AA of the rs109937179 polymorphism and rs208224995 allele CA is significantly higher than alleles CC and AA that is associated with an increased risk of mastitis.

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