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Identification of splice variants, expression analysis and single nucleotide polymorphisms of the *PRMT2* gene in dairy cattle

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ABSTRACT

Protein arginine N-methyltransferase 2 (*PRMT2*), also named *HRMT1L1*, belongs to the Bovine Protein arginine N-methyltransferase (*PRMT*) genes which are involved in the immune response. To explore the variability of the *PRMT2* gene and resistance to mastitis in cows, splice variant (SV), and single nucleotide polymorphisms (SNPs) were identified in this study. A SV (*PRMT2-SV*) lacking exon 7 (98-bp) of the *PRMT2* gene was found in healthy and mastitis-infected mammary gland tissues. Two of four SNPs were significantly associated with bovine milk yield and protein content. Further, we estimated the relative expression of *PRMT2-SV* in the mammary gland tissue of dairy cattle by using quantitative real-time polymerase chain reaction. The result showed that expression of the *PRMT2-SV* mRNA was significantly upregulated 4.02-fold ($p < 0.05$) in infected mammary tissues ($n = 5$) compared to healthy tissues ($n = 5$). Our findings reveal that *PRMT2-SV* may play an important role in mastitis resistance in dairy cattle. The SNPs may be used as a possible candidate SNPs for marker-assisted selection and management in Chinese Holstein cattle.

1. Introduction

Mastitis, an inflammation of the mammary gland, is a prevalent and complex infectious disease affected by genetics and pathogens that can result in significant economic losses of dairy cattle (Nash et al., 2003). Mastitis can be caused by many bacteria, including *Staphylococcus aureus* and *Escherichia coli*. The primary defense against pathogens relies on the appropriate expression of antigen presenting molecules triggering the release of effector molecules of the innate immune system. Thus, the innate immune system is an important line of defense against invading pathogens once they have penetrated the physical barrier of the streak canal, and it represents the primary host determinant for dictating the outcome of intramammary infection (Bannerman, 2009).

Protein arginine N-methyltransferases (*PRMTs*) are eukaryotic enzymes that catalyze the transfer of methyl groups from S-adenosylmethionine to arginine residues of numerous *PRMT* substrates (Bedford, 2007; Krause et al., 2007). Their activities influence a wide range of cellular processes, including cell growth (Lin et al., 1996), nuclear/cytoplasmic protein shuttling (McBride and Silver, 2001), differentiation and embryogenesis (Chen et al., 2002; Torres-padilla et al., 2007), RNA splicing and transport (Lukong and Richard, 2004; Meister and Fischer, 2002) and post-transcriptional regulation (Li et al., 2002). *PRMT2* (also known as *HRMT1L1*) belongs to the arginine methyltransferase family which is clearly involved in lung function (Yildirim et al., 2006), inflammatory response (Besson et al., 2007), apoptosis promotion (Ganesh et al., 2006), Wnt signaling and leptin signaling regulation (Blythe et al., 2010; Iwasaki et al., 2010) through different mechanisms.

Alternative splicing (AS) of eukaryotic pre-mRNAs is a key mechanism for potentially generating many transcript isoforms from a single gene, and this mechanism contributes greatly to phenotypic complexity (Kim et al., 2007). It serves versatile regulatory functions in controlling major developmental decisions and fine-tuning of gene function (Lopez, 1998). Many recent studies have pointed to the importance of detection and measurement of AS. For example, many immunologically related multiple-exon gene transcripts generated by AS play important roles in innate immunity (Lynch, 2004; Zhang et al., 2009; Zikherman and Weiss, 2008). The bovine *PRMT2* gene has been located on chromosome 1, and has 3 splice variants (SV) deposited in the Ensembl database (www.ensembl.org) which contained 8, 10 and 8 exons and encodes 279, 434, and 312 amino acids respectively. However, our knowledge of the

Abbreviations: AS, alternative splicing; aa, amino acids; Ne, effective allele numbers; He, heterozygosity; Ho, homozygosity; PCR-RFLP, PCR-Restriction Fragment Length Polymorphism; PCR-SSCP, PCR-single strand conformation polymorphism; PIC, polymorphism information content; PRMT2, protein arginine N-methyltransferase 2; Q-PCR, quantitative-PCR; RT-PCR, reverse transcription-polymerase chain reaction; SNP, single nucleotide polymorphism; SCC, somatic cell count; SCS, somatic cell score; SV, splice variant.

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Table 1
Primer sets for PCR and forced PCR-RFLP used for genotyping SNPs detected in bovine *PRMT2* gene.

SNPs[a]	Sequences of primers and position (5′–3′)	AT (°C)	SAF (bp)	PR	Genotype: bp
SNP1	CCTACCCTCCAAGTGGTTGT CGCTGCCCTTTTCACTAAGC	57.0	751	*TaqI*	TT: 751 CT: 751, 622, 129 CC: 622, 129
SNP2	CAGTCTCGTCCATTCTCTCG TGTCTTTCAGGGATTCTTTA	52.0	428	*Bsh1236I*	AA: 428 AG: 428, 392, 36 GG: 392, 36
SNP3	CACTCCTGGACGGATGCG ATGAGCCAAGGGCGGGTA	56.4	314	*NdeI*	CC: 314 CT: 314, 210, 104 TT: 210, 104
SNP4	CACTCCTGGACGGATGCG ATGAGCCAAGGGCGGGTA	56.4	314	*Hin1I*	TT: 314 CT: 314, 200, 114 CC: 200, 114

Note: AT annealing temperature, SAF size of amplification fragment, PR PCR–RFLP.
[a] SNPs: single nucleotide polymorphisms, SNP1 = C10202T, SNP2 = A10276G, SNP3 = C24375T, SNP4 = C24385T.

differential expression of specific splicing events and characterization of the *PRMT2* gene in mastitis resistance of cattle is limited.

The aims of this study were: (1) to investigate whether different SV of the *PRMT2* gene are present in different bovine tissues; (2) to analyze whether the *PRMT2* gene is differentially expressed in healthy and infected mammary gland tissue; (3) to explore genetic variants of the *PRMT2* gene; (4) to analyze the association between single nucleotide polymorphisms (SNPs) and milk production traits and mastitis.

2. Materials and methods

2.1. Animal samples

Samples were collected from five healthy and five mastitis-infected mammary gland tissues of first lactation Chinese Holstein cows from a commercial bovine slaughter farm. The initial selection of mastitis cows was based on clinical symptoms. One of the tissue samples was collected and stored in liquid nitrogen for RNA isolation; other tissues were collected and used for the identification of the pathogen. No pathogen was observed in the healthy cow's mammary tissues (n = 5). Only mammary tissues (n = 5) from *S. aureus* caused mastitis cases were used for this study. Mammary glands, heart, liver, spleen, lung, kidney, muscle, and intestine tissues from two healthy and two mastitis-infected cows were used for SV identification. All ten mammary tissue samples were used for analysis of the relative expression of *PRMT2* mRNA.

The blood samples of 548 multiparous Chinese Holstein dairy cattle at Xi'an Dairy Farm were collected from the jugular vein. All experimental protocols and animal care were performed according to authorization granted by the Chinese Ministry of Agriculture. The somatic cell count (SCC) and milk production trait (305-day milk yield, fat content and protein content) data of sampled individuals were collected monthly from the beginning of the 2nd lactation to the end of 5th lactation, they were measured ten times per lactation (305 days). Each blood sample was placed in a tube with acid–citrate–dextrose anticoagulant for genomic DNA extraction. The DNA content was estimated spectrophotometrically, and the genomic DNA was diluted to 50 ng/mL. DNA samples were stored at −20 °C for subsequent analysis.

2.2. Reverse transcription-polymerase chain reaction

Total RNA was extracted from the mammary tissue using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. RNA integrity was assessed by electrophoresis on 1.2% agarose gel. RNA purity was verified by measuring the absorbance at 260 and 280 nm by ND-1000 spectrophotometer (NanoDrop Technologies, USA) and cDNA for each sample was synthesized from equal amount of total RNA (500 ng) by PrimeScript RT reagent kit (TaKaRa, Japan) following the manufacturer's protocol.

To identify SV of the *PRMT2* gene, primers were designed for reverse transcription-polymerase chain reaction (RT-PCR) amplification based on the coding sequence of mRNA in GenBank (NM_001024495 and BT021771.1). The primers used were as follows: *PRMT2*-F, 5′-AGGTGA CTGCCCCAGAAATG-3′; *PRMT2*-R, 5′-ACGCATCCGTCCAGGAGTGA-3′ (from Exon1 to 3′-UTR). PCR reactions were performed with a total 20 μL in each tube, each containing 2 μL 10 × PCR buffer (Mg^{2+} plus), 50 ng cDNA, 0.25 μM of each primer, 0.25 μM of each dNTP and 2.0 U Tap DNA polymerase (MBI Fermentas, USA). Conditions for amplification were 95 °C for 5 min, followed by 30 cycles of 94 °C, 30 s; 55 °C, 30 s; and 72 °C, 1.5 min. Reactions were finished with a 72 °C, 10-min extension. PCR products were gel-purified, ligated into the pMD18-T Easy vector (TaKaRa, Japan) and then transformed into competent *E. coli* DH5α. Fifteen clones were selected at random and subjected to DNA sequencing.

2.3. Quantitative real-time PCR

To determine the relative expression of the *PRMT2* SV, quantitative-PCR (Q-PCR) was carried out using the SYBRGreen PCR Master Mix (TakaRa, Japan) according to the manufacturer's protocol. The primers of the target bovine *PRMT2*-SV gene (F: 5′-AGAATGGCTTTGCTGATA-3′; R: 5′-GGGCATACAAGATGGACT-3′; size = 144-bp), and housekeeping internal control gene *GADPH* (GenBank accession no. NC_007303; primer sequences, F: 5′-ATCATCTCTGCACCTTCTGCCGAT-3′; R: 5′-TAAGTCCCTCCACGATGCCAAAGT-3′; size = 166-bp) were designed using Primer5 software (Premier Biosoft International, Palo Alto, USA). The Q-PCR protocol and calculation of relative expression were described by Schmittgen and Livak, (2001).

2.4. PCR amplification and DNA sequencing analysis

Primers used to amplify cattle *PRMT2* gene were designed by Primer5 software, according to the published gene sequence (GenBank accession number AC_000158). Primers, restriction enzymes selected (ABI, Foster City CA) and fragment sizes were listed in Table 1. The detection results of allelic variation were based on the electrophoretic pattern of the restriction enzyme-treated PCR products.

PCR amplifications were performed in a total volume of 20 μL, where the volume mixture contained: 50 ng of genomic DNA as template, 2 μL 10 × PCR buffer (Mg^{2+} plus), 0.25 μM of each primer, 0.25 μM of each dNTP and 2.0 U Tap DNA polymerase (MBI Fermentas, USA). PCR conditions were as follows: after an initial denaturation of 5 min at 95 °C, amplicons were generated for 35 cycles of 30 s at 94 °C, 30 s at an optimal annealing temperature, and 45 s at 72 °C, followed by a 10 min final extension at 72 °C.