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ORIGINAL PAPER

DNA methylation rather than single nucleotide polymorphisms regulates the production of an aberrant splice variant of *IL6R* in mastitic cows

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Abstract

Interleukin-6 receptor-alpha (IL6R) interacts with IL6 and forms a ligand–receptor complex, which can stimulate various cellular responses, such as cell proliferation, cell differentiation, and activation of inflammatory processes. Both genetic mutation and epigenetic modification regulate gene transcription. We identified a novel splice variant of bovine *IL6R*, designated as *IL6R-TV*, which is characterized by the skipping of exon 2 of the NCBI-referenced *IL6R* gene (*IL6R-reference*). The expression levels of *IL6R-TV* and *IL6R-reference* transcripts were lower in normal mammary gland tissues. These transcripts play a potential role during inflammatory infection. We also detected two putative functional SNPs (g.19711 T > C and g.19731 G > C) located within the upstream 100 bp of exon 2. These SNPs formed two haplotypes (T-G and C-C). Two mutant pSPL3 exon-trapping plasmids (pSPL3-T-G and pSPL3-C-C) were transferred into the bovine mammary epithelial cells (MAC-T) and human embryonic kidney 293 T cells (HEK293T) to investigate the relationship between the two SNPs and the aberrant splicing of *IL6R*. DNA methylation levels of the alternatively spliced exon in normal and mastitis-infected mammary gland tissues were quantified through nested bisulfate sequencing PCR (BSP) and cloning sequencing. We found that DNA methylation regulated *IL6R* transcription. The DNA methylation level was high in mastitis-infected mammary gland tissues and stimulated *IL6R* expression, thereby promoting the inclusion of the alternatively spliced exon. The upregulated expression of the two transcripts was due to DNA methylation modification rather than genetic mutations.

Keywords *IL6R* · Splice variant · Single nucleotide polymorphism · Cattle · Mastitis · DNA methylation

Yan Zhang and Xiuge Wang contributed equally to this work.

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Abbreviations

IL6R	Interleukin-6 receptor-alpha
SNPs	Single nucleotide polymorphisms
MAC-T	Bovine mammary epithelial cells
HEK293T	Human embryonic kidney 293 T cells
BSP	Bisulfate sequencing PCR
E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
ASEs	Alternatively spliced exons
TPI	Total performance index
SCS	Somatic cell score
Q-PCR	Quantitative real-time PCR
D M E M -F12	Dulbecco's modified Eagle medium/Ham's F-12 medium
DMEM	Dulbecco's modified Eagle medium
TFBS	Transcriptional factor binding sites
ESE	Exonic splicing enhancer

DMRs	Differentially methylated regions
E T S family	E26 transformation-specific family
mIL6R	Membrane-bound interleukin-6 receptor-alpha
sIL6R	Soluble interleukin-6 receptor-alpha
SR family	Serine/arginine (SR) family

Introduction

Clinical mastitis is a visible inflammatory disease that infects the mammary gland of dairy cows and the most frequent disease of dairy cattle which can be potentially fatal. Bovine mastitis is an economically important pathology associated with reduced milk production and changes in milk composition and quality (Gomes and Henriques 2016), and is considered the most costly infectious disease in dairy cows (Rainard et al. 2016). Many programs have been developed to improve bovine resistance and reduce the occurrence of mastitis. However, conventional strategies based on phenotypic selection are inefficient because of their low heritability (Hinchs et al. 2011). By contrast, genomic selection programs are an effective tool against diseases and important for cattle breeding (Wiggans et al. 2016; García-Ruiz et al. 2016).

Inflammation is a biological process commonly caused by bacterial infection and initiated upon injury to remove harmful stimuli and induce healing. However, prolonged inflammation may be detrimental to the host and promote a permanent disease state, resulting in gene expression dysregulation and tissue damage (Huang et al. 2014). Bovine mastitis leads to the overwhelming release of pro-inflammatory cytokines, which play pivotal roles in regulation of inflammatory responses, including increasing macrophage and neutrophil bactericidal capacity, promoting the recruitment of neutrophils towards the site of infection in the pathophysiology of bovine mastitis, inducing the maturation of dendritic cells, and controlling the acquired immune response (Oviedo-Boyso et al. 2007). IL6 is the keystone cytokine and modulates almost every aspect of the innate immune system; it promotes an alternatively activated macrophage phenotype associated with wound healing and inhibits the microbicidal activities of macrophages and the production of pro-inflammatory cytokines (Hunter and Jones 2015a).

Escherichia coli and *Staphylococcus aureus* pathogens can rapidly enhance *IL6* expression (Günther et al. 2011). Interleukin-6 receptor-alpha (IL6R), a receptor of IL6, was upregulated in mastitic udder tissues challenged by bovine *E. coli* 24 h postinfection (Buitenhuis et al. 2011). The expression level of *IL6R* increased by 17-fold in lipopolysaccharide-challenged human mammary epithelial cells (Wang et al. 2013a). By contrast, *IL6R* expression was downregulated by 3.53-fold in bovine monocyte-derived macrophages infected with *S. aureus* in vitro 6 h postinfection (Lewandowska-Sabat et al. 2013). Thus far, the molecular mechanism underlying *IL6R* expression in dairy cattle remains unknown.

Alternative gene splicing is a universal phenomenon that enables a single coding gene to generate many products with similar or different functions (Brett et al. 2001). Several important diseases have been linked with mutations or variations in either cis-acting elements or trans-acting factors that lead to aberrant splicing and abnormal protein production (Garcia-Blanco et al. 2004). These variants, such as SNPs, affect gene function and induce a disease phenotype because the splice mutation shifts the protein reading frame. Commonly observed alternative splicing events preserve the reading frame and minimally affect the protein function (Resch et al. 2004). Our previous studies showed that several immune-related genes undergo alternative splicing events, and SNPs located in splicing-related domains influence susceptibility to bovine mastitis (Wang et al. 2014; Ju et al. 2015; Zhang et al. 2015). However, individuals with the same genetic background may develop different levels of disease severity; this variation cannot be explained by genetics alone; one of the many potential nongenetic sources of variation is epigenetic modification (Runyon et al. 2012; Tryndyak et al. 2016).

Epigenetic modification includes acetylation and methylation of histone and methylation of long noncoding RNAs and DNA (Breiling and Lyko 2015; Larriba and del Mazo 2016; Messier et al. 2016). Of these processes, DNA methylation is the most widely studied because it plays an important role in genome-wide pre-transcriptional regulation (Song et al. 2016), such as alternative splicing (Yearim et al. 2015; Lev Maor et al. 2015). DNA methylation is altered by bacteria (Benakanakere et al. 2015) or inflammation caused by infection (Niwa et al. 2010). Researchers determined that bovine mastitis increased the methylation levels in the promoter region of *CD4* and decreased the expression level of *CD4* by inhibiting the binding of transcription factors (Wang et al. 2013b). Moreover, mastitis caused by *E. coli* induced the loss of promoter methylation and increased the expression level of the *TLR4* gene (Chang et al. 2015). These studies confirmed the role of DNA methylation in innate immune responses in the process of mastitis. Perturbation of DNA methylation leads to the aberrant splicing of alternatively spliced exons (Yearim et al. 2015; Maunakea et al. 2013). Thus, the generation of new splice variants can be influenced by both genetic and epigenetic mechanisms.

This study aims to determine (1) whether various splice variants of *IL6R* are commonly observed in the mammary glands of normal and mastitic cows, (2) whether genetic variants that affect splicing patterns are located within the splicing sites, and (3) whether epigenetic modification regulates the expression and alternative splicing patterns of *IL6R*.

Materials and methods

Animal and tissue samples

Mammary tissue samples were collected from three normal and three mastitis-infected first-lactating Holstein cows obtained from a commercial bovine slaughterhouse in Jinan, Shandong Province, China. The cows were classified into normal and mastitic groups based on clinical symptoms and pathogenic bacteria from the milk culture test. The normal cows did not present any clinical symptoms, such as heat, pain, redness, swelling of the udder, or milk clotting, and were not infected by pathogens. Clinically diagnosed mastitis cows were identified based on these clinical signs. The milk isolates of bovine mastitis mammary areas and the biopsies of mammary gland tissues were tested for pathogenic bacteria through cell culture (Wang et al. 2014; Ju et al. 2015). A total of 18 tissue samples (three for each cow) were immediately frozen in liquid nitrogen. DNA was extracted from six tissue samples by using a TIANamp Genomic DNA Kit (Tiangen, Cat. #DP304) and stored at − 20 °C. Total RNA was extracted from six mammary tissues by using an RNAsimple Total RNA Kit (Tiangen, Cat. #DP419) according to the manufacturer's instructions. RNA concentration was determined with a Nanophotometer (P330; IMPLEN). RNA and DNA quality was monitored by visualization of ethidium bromide-stained bands in 1% agarose gels after electrophoresis. The RNA samples were stored at − 80 °C.

Sixty-eight Holstein bulls (aged between 1 and 3 years old) with genomic estimated breeding values (gEBVs) were selected from Shandong OX Livestock Breeding Co., Ltd. These bulls were unrelated individuals, which were specially chosen according to the pedigrees. The bulls were obtained by embryo transfer of frozen embryo from North America and genotyped by the bovine GH2 SNP chips (Illumina, GeneSeek 77k chip, version 2), and their genetic evaluations are completed by the Holstein Association USA (http://www.holsteinusa.com/). A gEBV includes mastitis-related traits, such as somatic cell score (SCS) and udder conformation, and may be used for association analysis. Semen samples were collected from the 68 bulls, and DNA was extracted according to the protocol in our previous study (Gao et al. 2014). All DNA samples were stored at − 20 °C for subsequent analysis.

Identification of *IL6R* novel splice variant

RNA samples were reverse transcribed into cDNA with the PrimeScript™ II First-strand cDNA Synthesis Kit (TaKaRa, Cat. #6210A). RT-PCR analysis was performed with specific primers of IL6R-cDNA (Additional file: Table S1) to amplify the coding region of the *IL6R* gene by using 2× Taq Master mix (Generay, Cat. #GK8006) according to the manufacturer's instructions. After electrophoresis, the products were purified with a TIANgel Midi Purification Kit (Tiangen, Cat. #DP209), cloned into the pEASY-T3 vector (Transgene, Cat. #CT301), and transformed into Trans5α (Transgene, Cat. #CD201). Positive clones were directly sequenced by a commercial sequencing company (BGI). The results were compared with the reference *IL6R* mRNA sequence (GenBank, NM_001110785.1) by using DNAMAN software. Clustal OMEGA (http://www.ebi.ac.uk/Tools/msa/clustalo/) was applied to align the sequences.

Expression analysis of splice variants

Quantitative real-time PCR (qRT-PCR) analysis was conducted to investigate the relative expression of *IL6R* transcripts in normal and mastitis-infected bovine mammary gland tissues. Analysis was performed using SYBR® Premix Ex Taq™ (TliRNaseH Plus) (TaKaRa, Cat. #RR420A) on a Roche LightCycler® 480II machine (Roche Applied Science). The qRT-PCR protocol and calculation of relative expression were described in our previous study (Ju et al. 2015). Two pairs of specific primers IL6R-G and IL6R-TV (Additional file: Table S1) were used to quantify the expression of *IL6R-reference* mRNA and the novel splice variant *IL6R-TV* mRNA. The housekeeping gene *β-actin* (Additional file: Table S1) was used as reference to normalize data (Hou et al. 2012). The template that did not undergo the reverse transcription reaction served as negative control. All qRT-PCRs were performed in triplicate. Inclusion levels of exon 2 were calculated by dividing the amount of *IL6R-reference* by the amount of *IL6R-TV (Yearim et al. 2015)*. The relative expression of the two splice variants (*IL6R-reference* and *IL6R-TV*) and the relative exon inclusion level were compared between the normal and mastitis-infected cows by using two-tailed, unpaired Student's *T* test. Differences with $P < 0.05$ were considered statistically significant.

Screening, genotyping of SNPs, and statistical analysis

The fragments encompassing the exon 2 of *IL6R* were amplified using the specific primer EXON2 (Additional file: Table S1). The PCR products of 68 semen samples and six cows were sequenced. Two SNPs located upstream of exon2 were in complete linkage disequilibrium. The two SNPs formed two haplotypes (T-G and C-C) in the studied bulls. SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) was used to analyze the pairwise linkage disequilibrium and haplotypic frequency (Gao et al. 2014). The association

between genotypes and gEBVs was tested by Student's *T* test. Values with $P < 0.05$ were considered significant.

Splicing mini-gene reporter assay

Construction of vectors The 804-bp genomic fragments spanning 369 bp of intron 1, 249 bp of exon 2, and 186 bp of intron 2, which harbored the two SNPs, were amplified using the specific primer E2 (Additional file: Table S1). The mini-gene reporter assays were constructed by cloning the 804-bp fragments with the wild type (g.19711 T > C and g.19731 G > C; wild haplotype: T-G) or the mutant type (g.19711 T > C and g.19731 G > C; mutant haplotype: C-C) into the empty pSPL3 vector after digestion with *EcoR*I and *Xho*I. The constructed vectors and the empty vector were transformed into Trans5α. The clones were cultured on agar containing 100 mg/mL ampicillin and incubated overnight at 37 °C. Positive clones were chosen and incubated overnight at 37 °C in 10 mL of lysogeny broth medium containing 100 mg/mL ampicillin. Plasmids were isolated with the EZNA Endo-Free Plasmid DNA Midi Kit (OMEGA, Cat. #D6915) and then measured by a NanoPhotometer (P330; IMPLEN). The plasmids were sequenced after isolation to guarantee the authenticity and orientation of the vectors.

Cell culture Bovine mammary epithelial cells (MAC-T) and human embryonic kidney 293 T cells (HEK293T) were used in our experiments. Dulbecco's modified Eagle medium/Ham's F-12 medium (DMEM-F12) (GIBCO, Cat. #1699748) was used as the basic medium for MAC-T, and Dulbecco's modified Eagle medium (DMEM) (GIBCO, Cat. #1744353) was used as the basic medium for HEK293T. The growth medium comprised the basic medium supplemented with 500 U/L penicillin, 500 mg/L streptomycin (GIBCO, Cat. #15140163), and 10% (*v*/*v*) fetal bovine serum (GIBCO, Cat. #16000044). Both cells were maintained at 37 °C with 5% CO_2 and subcultured every other day.

Transfection MAC-T and HEK293T were used for transfection. The cells were cultured in six-well culture plates. One day before the transfection, the medium was replaced by removing the antibiotics. When the cells reached 80% confluence, 4 μg of the mini-gene reporters or pSPL3 vector was added to the Lipofectamine 2000 Transfection Reagent (Invitrogen, Cat. #11668030) in Opti-MEM medium (Gibco, Cat. #51985042). A growth medium without antibiotics was substituted for the Opti-MEM medium after 5 h. The cells were harvested 24 h after transfection, and total RNA was extracted with the RNAsimple Total RNA Kit (Tiangen, Cat. #DP419) to determine the expression of mini-gene reporters.

Expression analysis of splice variants RNA extracted from the transfected cells was reverse transcribed into cDNA by using the pSPL3 vector-specific primer SA2 with the PrimeScript™ II First-strand cDNA Synthesis Kit (TaKaRa, Cat. #6210A). Specific primers for pSPL3 vector SD6 and SA2 were used to perform RT-PCR as previously described. The products were separated through electrophoresis on 1% agarose gels. Each DNA band was further analyzed by direct sequencing after extraction with the TIANgel Midi Purification Kit (Tiangen, Cat. #DP209).

Analysis and prediction of CpG island

The online software Methprimer (http://www.urogene.org/cgi-bin/methprimer/ methprimer.cgi) was used to detect and analyze the CpG island located within exon 2 of the *IL6R* gene.

Bisulfite treatment

A total of 200 ng of genomic DNA from the six mammary tissue samples collected from three normal and three mastitis-infected cows was used for bisulfate modification with a BisulFlash DNA Modification Kit (Epigentek, Cat. #P-1026) according to the manufacturer's instructions. DNA was stored at − 20 °C until use as templates for bisulfate sequencing PCR (BSP).

Nested BSP

Two special pairs of primer (BSP-F2/R2 for the first step and BSP-F1/R1 for the second step) were designed using Methyl Primer Express Software v1.0. PCR analysis was conducted using 5 μL of 2× GC buffer (5 mM Mg^{2+} Plus, TaKaRa, Cat. #9154), 1.6 μL of 2.5 mM dNTP mixture (TaKaRa, Cat. #4030), 0.1 μL of TaKaRa LA Taq (5 U/μL, TaKaRa, Cat. #RR52A), 0.5 μL of each primer (10 mM), and 0.5 μL of template DNA in a total volume of 10 μL. The first step of PCR was performed as follows: (1) 94 °C denaturation for 5 min, (2) 16 thermal cycles of denaturation at 94 °C for 30 s, (3) annealing starting at 56 °C and reducing 1 °C every cycle for 30 s, (4) extension at 72 °C for 1 min, and (5) another 29 cycles of denaturation at 94 °C for 30 s, 41 °C for 30 s, and 72 °C for 1 min. Final extension was conducted for 10 min at 72 °C. The products of the first step were used as templates for the second step, which was conducted as follows: (1) 94 °C denaturation for 5 min, (2) followed by 16 thermal cycles of denaturation at 94 °C for 30 s, (3) annealing starting at 55 °C and reducing 1 °C every cycle for 30 s, (4) extension at 72 °C for 1 min, and (5) another 29 cycles of denaturation at 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 1 min. Final extension was performed for 10 min at 72 °C. The PCR products were purified, cloned into pEASY-T3 vectors, and transformed into

Trans5α for sequencing. Sequences with > 95% cytosine conversion were analyzed through BiQ Analyzer software with the reference *IL6R* gene sequence.

Results

Identification of a novel bovine *IL6R* splice variant

Six mammary tissues of dairy cows (three healthy cows and three mastitic ones) were used to amplify the entire coding region of the reference *IL6R* gene. In addition to the predicted 664-bp PCR product, a smaller band (415 bp) was detected (Fig. 1a). After the cloning and sequencing of the smaller band, a new transcript, named *IL6R-TV*, was found in both normal and mastitic mammary gland tissues. The novel *IL6R-TV* (415 bp) was characterized by the skipping of the entire exon 2 (Fig. 1b); therefore, the exon 2 of the *IL6R* gene was considered an alternatively spliced exon.

Bioinformatic analysis suggested that the IL6R-reference and IL6R-TV proteins shared the same signal peptide and transmembrane domain. In contrast to the IL6R-reference protein, the putative IL6R-TV protein lost 83 amino acids encoding an immunoglobulin-like domain (Additional file: Fig. S1).

Analysis of the expression of the two transcripts in mammary tissues

Quantitative real-time PCR (qRT-PCR) results showed that the two transcripts were expressed in the mammary tissues of normal and mastitic cows; the *IL6R-reference* was found to be the primary transcript in the mammary gland tissues (Fig. 2a).The expression levels of *IL6R-reference* and *IL6R-TV* in the normal mammary tissues were significantly lower than those in the mastitic tissues ($P < 0.05$). The inclusion levels of exon 2 were significantly higher in the mammary tissues of mastitis-infected cows than those in normal cows ($P < 0.05$) (Fig. 2b).

Putative effect of SNPs on splicing in exonic splicing enhancer motif

We amplified the genomic region from exon 1 to exon 3 of the bovine *IL6R-reference* gene by using specific primers EXON2. We directly sequenced the PCR products with specific primer E2F and E2R in 68 bulls and six cows and found two SNPs (g.19711 T > C and g.19731 G > C), which were located 85 and 65 bp upstream of exon 2 of *IL6R*, respectively (Fig. 1a), and within the exonic splicing enhancer (ESE) motif region. ESEfinder 3.0 (http://www.mybiosoftware.com/esefinder-3-0-exon-splicing-enhancer-finder.html) was applied to identify exonic splicing enhancer motifs for wild and mutant sequences. Two binding sites for the splicing factors SF2/ASF and SRp40 diminished, and one binding site for the splicing factor SC35 was added to the mutant (Additional file: Figs. S2 and S3). Genetic mutations can putatively affect the binding of the splicing factors. However, the effect of changes in the binding of splicing factors on the splicing patterns of *IL6R* was not determined in this study.

Cell culture and transfection

The SV-40 T transformed bovine MAC-T cell line was an easy-to-handle model for the analysis of immune functions of mammary epithelial cells (Günther et al. 2016). So MAC-T was applied in our experiments; HEK293T was chosen as the reference. We constructed two *IL6R* mini-genes which comprised the wild and mutant haplotypes of the two SNPs and transfected them into two cell lines, namely MAC-T and HEK293T, respectively, to investigate their effects at the splicing level and investigate whether SNP genotypes directly define the patterns of the spliced isoforms (Fig. 3).The RT-PCR results of the splicing assay showed that both wild haplotype (T-G) and mutant haplotype (C-C) mini-gene vectors produced a 512-bp fragment, which included the 249 bp of exon 2 and 263 bp of pSPL3. The empty pSPL3 vector generated a 263-bp fragment and a nonspecific binding fragment in MAC-T (Fig. 3d) and HEK293T (Fig. 3e). In both cells, the small RT-PCR product corresponding to the mini-genes of the two haplotypes was directly sequenced and showed nonspecific binding. Both the wild and mutant haplotype reporters did not generate the predicted 263-bp fragment (Fig. 3d, e). This finding indicated that the two haplotypes did not produce the novel splice variant *IL6R-TV* characterized by deleting the entire exon 2.

Association between two SNPs and the gEBV of the SCS as well as udder conformation

Somatic cell count (SCC) and SCS are used as indicators of dairy mastitis (Wang et al. 2014). Lund et al. (Lund et al. 1994) showed that improved udder conformation reduced the SCC and the occurrence of clinical mastitis. SCC, SCS, and udder conformation can be considered as indicators of dairy mastitis. Sixty-eight bull samples were used to amplify the fragments containing the two SNPs, and the bulls were genotyped through sequencing to determine the association of the SNPs with mastitis in dairy cows. The linkage disequilibrium between the two SNPs in the population was complete: there were only two haplotypes, namely T-G and C-C. The two haplotypes resulted in three two-locus genotypes, namely TT-GG, TC-GC, and CC-CC, with frequencies 60.3, 38.2, and 1.5%, respectively. The relationship between the three genotypes and mastitis indicators, including gEBVs of SCS and seven udder traits, was analyzed. Since only one sample

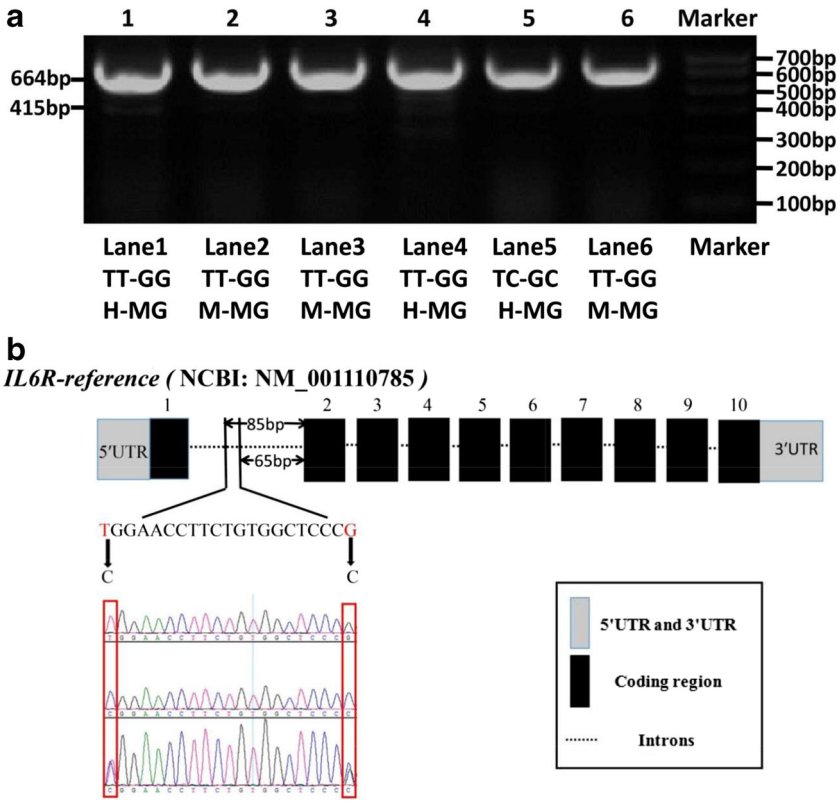


Fig. 1 RT-PCR products from the *IL6R* gene expressed in bovine mammary tissues and schematic representation of the splicing patterns of *IL6R* transcripts and SNPs (g.19711 T > C and g.19731 G > C): **a** RT-PCR products from the *IL6R* gene expressed in bovine mammary tissues; lane 1–lane 6: RT-PCR products of different cows. TT-GG and TC-GC indicated the genotype combination of the cows. H-MG, healthy mammary glands. M-MG, mastitis mammary glands. **b** Genomic structure of the bovine *IL6R* gene and *IL6R-TV.* The *IL6R-TV* splice variant lacks the sequence of exon 2. Primers E2F and E2R span exon 2 and are used to amplify fragments to screen the existence of SNPs. The position of the A nucleotide in the start codon (ATG) is defined as +1

of genotype CC-CC was detected, this genotype was not included in the association analysis. The results indicated that the two SNPs were not significantly associated with SCS ($P > 0.05$); hence, the two SNPs might not be used as the markers for resistance to mastitis (Table 1). More samples are needed to further investigate their correlation.

Analysis of DNA methylation status

DNA methylation affects pre-mRNA splicing by influencing the recognition of an alternatively spliced exon (Yearim et al. 2015; Maunakea et al. 2013). In the present study, the second exon and intron of *IL6R* which spanned one CpG island was identified by Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Fig. 4a). The result indicated the presence of epigenetic regulation by ASE methylation. Nested BSP and touchdown PCR analyses were used to amplify the 233 bp of the CpG island with 13 CpG sites. Sixty clones (10 clones for each cow) were selected for analysis. The methylation rate significantly differed and was found to be higher in the mammary gland tissues of mastitis-infected cows than in normal cows (Fig. 4b, c).

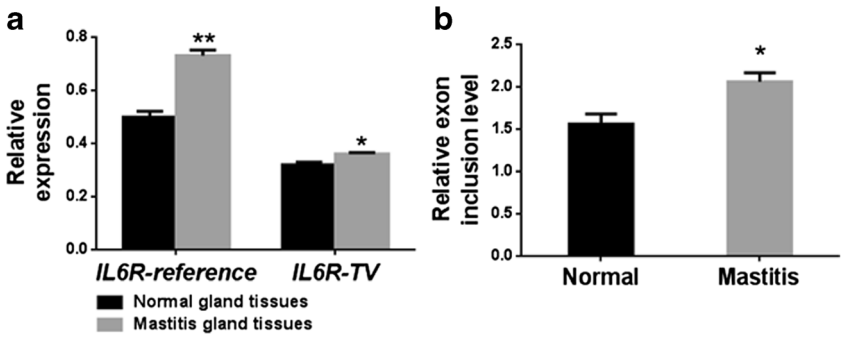
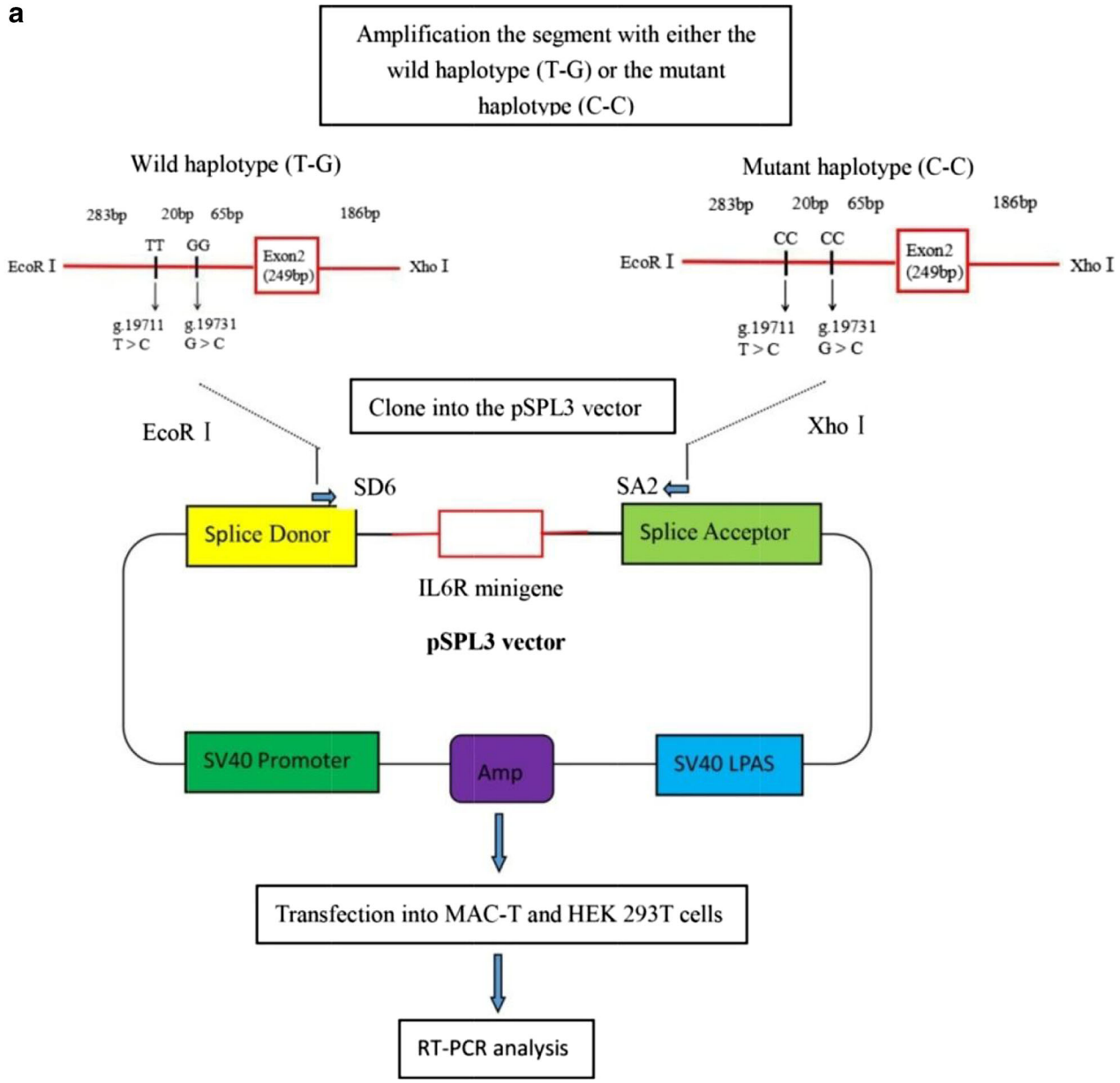
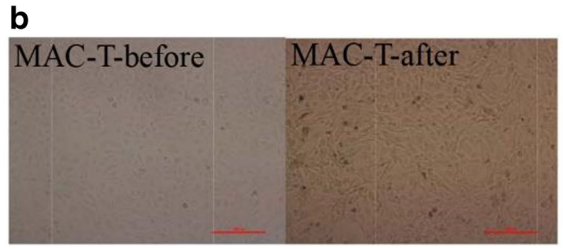
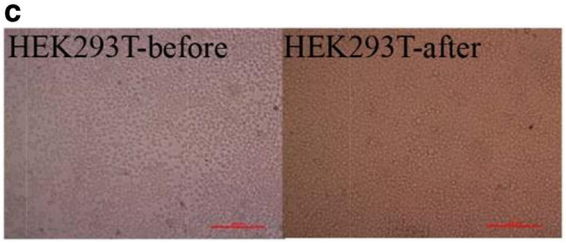
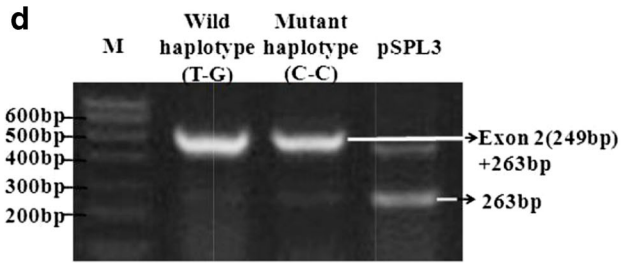


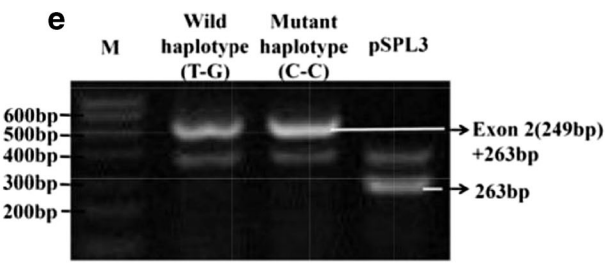
Fig. 2 Expression levels of different transcripts of *IL6R* and the relative exon inclusion levels between normal and mastitis-infected groups. **a** qRT-PCR of *IL6R-reference* and *IL6R-TV* of normal and mastitis-infected cows. One asterisk, $P < 0.05$, significantly different from normal controls. Two asterisks, $P < 0.01$, significantly different from normal controls. **b** The relative exon inclusion level was calculated by dividing the amount of the inclusion isoform with the amount of the skipping isoform. Asterisk, $P < 0.05$, significantly different from normal controls


a
Amplification the segment with either the wild haplotype (T-G) or the mutant haplotype (C-C)
Wild haplotype (T-G)
283bp
20bp
65bp
186bp
TT
GG
EcoR I
Exon2 (249bp)
Xho I
g.19711 T>C
g.19731 G>C
Mutant haplotype (C-C)
283bp
20bp
65bp
186bp
CC
CC
EcoR I
Exon2 (249bp)
Xho I
g.19711 T>C
g.19731 G>C
Clone into the pSPL3 vector
EcoR I
Xho I
SD6
SA2
Splice Donor
Splice Acceptor
IL6R minigene
pSPL3 vector
SV40 Promoter
Amp
SV40 LPAS
Transfection into MAC-T and HEK 293T cells
RT-PCR analysis


b
MAC-T-before
MAC-T-after


c
HEK293T-before
HEK293T-after


d
M
Wild haplotype (T-G)
Mutant haplotype (C-C)
pSPL3
600bp
500bp
400bp
300bp
200bp
Exon 2(249bp) +263bp
263bp


e
M
Wild haplotype (T-G)
Mutant haplotype (C-C)
pSPL3
600bp
500bp
400bp
300bp
200bp
Exon 2(249bp) +263bp
263bp

◀ **Fig. 3** Two haplotypes generated the same splicing pattern of *IL6R* in MAC-T and HEK293T: **a** protocol of splicing mini-gene reporter assay. **b** Microscopic images of MAC-T before and after cell transfection with two IL6R mini-genes (amplification, 10 × 20). **c** Microscopic images of HEK293T before and after cell transfection with two IL6R mini-genes (amplification, 10 × 20). **d** Splicing mini-gene reporter assay in MAC-T. **e** Splicing mini-gene reporter assay in HEK293T. The wild haplotype (g.19711 T > C-T and g.19731 G > C-G; haplotype: T-G) and the mutant type (g.19711 T > C-C and g.19731 G > C-C; haplotype: C-C) mini-gene reporters give rise to a 512-bp fragment, which consists of exon 2 and the 263-bp fragment of pSPL3 vector in the MAC-T and HEK293T. Nonspecific amplification products were visible and sequenced

Further analysis showed that the methylation levels of the 3rd, 6th, 8th, 9th, and 10th CpG sites were significantly higher in mastitic cows than in normal cows (Fig. 4d).

Discussion

IL6 has long been recognized as a prototypic pro-inflammatory cytokine that is involved in the pathogenesis of all inflammatory diseases (Garbers et al. 2015). IL6 acts on the immune system by regulating the development and activation status of innate and adaptive immune cells (Fontes et al. 2015). As the receptor of IL6, IL6R stimulates various cellular responses, including inflammatory processes, to initiate intracellular signaling pathways by participating in the formation of the signal transducing complex IL-6/IL6R/gp130 (Hunter and Jones 2015b; Kumar et al. 2015). IL6 first binds to IL6R and forms a complex, which binds to gp130 to initiate different pathways, such as those of MAPK-C/EBPβ and JAK/STAT (Kumar et al. 2015). Human IL6R, which exists in membrane-bound (mIL6R) and soluble (sIL6R) forms, plays critical roles in eliciting immune responses (Wang et al. 2013a; Garbers et al. 2015; Lopez-Lasanta et al. 2015). This protein is expressed on normal hepatocytes, $CD4^+$ or $CD8^+$ T cells (Jones et al. 2011), inflammatory cells, and cancer cells (Zhang et al. 2013; Fenton et al. 2000). Human sIL6R is one of the *IL6R* transcripts partly generated by the alternative mRNA splicing of the *IL6R* gene by skipping exon 9 (Li et al. 2015). The *IL6R* gene, also known as *IL6Rα*, *CD126*, and *gp80* gene, possesses other splice variants generated through alternative or aberrant splicing in other species, such as mice and pigs (http://ensemblgenomes.org/). Thus, *IL6R* expression and its regulation should be investigated to elucidate its potential role in bovine mastitis.

In this study, a novel *IL6R* splice variant without the entire exon2 of the *IL6R-reference* gene, named *IL6R-TV*, was identified. This study is the first to report a distinct alternative splicing pattern of the bovine *IL6R* gene. Furthermore, two transcripts, *IL6R-reference* and *IL6R-TV*, were high-regulated in the mastitic tissues. *IL6R* was upregulated in *E. coli*-challenged bovine udder tissues 24 h postinfection (Buitenhuis et al. 2011) and in lipopolysaccharide-challenged human mammary epithelial cells at the early stage of infection (Wang et al. 2013a). *IL6R* was downregulated by 3.53-fold in bovine monocyte-derived macrophages infected with *S. aureus* in vitro 6 h postinfection (Lewandowska-Sabat et al. 2013). In the present study, the expression levels of *IL6R-reference* and *IL6-TV* increased in the mammary tissues of mastitic cows, suggesting their potential role against pathogenic infection.

Inflammation is a normal and essential immunological process to defend the host against pathogens. Host inflammatory responses eliminate the source of tissue injury, restore immune homeostasis, and return affected mammary glands to normal function. Imbalance between pro-inflammatory and pro-resolving mechanisms is needed to ensure optimal bacterial clearance and induce return to immune homeostasis (Aitken et al. 2011). Therefore, changes of the *IL6R* expression level are necessary in the process of inflammation. The IL6R gene may be considered as a candidate gene for mastitis susceptibility.

Numerous SNPs are located in splicing-relevant sequences, such as canonical splice-site consensus sequences, including ESE, intronic splice enhancer, exonic splicing silencer, and intronic splicing silencer (ElSharawy et al. 2006). An increasing body of evidence demonstrated the functional importance of alternative splice variations and splicing-related SNPs within intronic and exonic motif sequences in mastitis (Wang et al. 2014; Ju et al. 2015; Zhang et al. 2015; Li et al. 2013). Thus, we searched genetic mutation within the close gene sequences around *IL6R* splicing sites. The results revealed two SNPs (g.19711 T > C and g.19731 G > C) in the upstream of the missed exon 2. Analysis of bioinformatic prediction suggested that the two SNPs were located in the ESE motif and influenced the target region-binding capability of splicing-related factors. The mutant alleles abolished two binding sites for the splicing factors SF2/ASF and SRp40 and increased one binding site for the splicing factor SC35. The serine/arginine family of proteins constitutes an essential class of splicing regulators through splice-site selection. Mammalian SF2/ASF binds to a purine-rich motif and coordinates with SRSF2 to regulate exon inclusion and skipping (Bradley et al. 2015). SRp40 and SC35 splicing factors are associated with the formation of gene splice variants characterized by variable exons (Bradley et al. 2015; Huang et al. 2007). Through in silico prediction, we initially speculated that the two SNPs might be the source of aberrant splice variant *IL6R-TV*. Therefore, a pSPL3 exon-trapping system was used to test this hypothesis. We constructed two mini-gene vectors with different haplotypes

Table 1 Association between different combined genotypes and gEBVs of SCS and udder conformation in Holstein bulls

Genotype combination	Sample number	Frequencies of genotype combination (%)	Haplotypic frequencies (%)	SCS gEBV	FUA gEBV	RUH gEBV	UC gEBV	UDE gEBV	FTP gEBV	RTP gEBV	FTL gEBV
TT-GG	41	60.29	79.41 (T-G)	2.90 ± 0.12	1.25 ± 0.78	1.73 ± 0.85	0.71 ± 0.58	0.73 ± 0.64	0.62 ± 0.64	0.58 ± 0.74	− 0.32 ± 1.03
TC-GC	26	38.24		2.93 ± 0.12	1.02 ± 0.67	1.37 ± 0.67	0.66 ± 0.83	0.70 ± 0.85	0.68 ± 0.72	0.72 ± 0.83	− 0.17 ± 0.69
CC-CC	1	1.47	20.59 (C-C)	2.70	1.56	1.94	0.70	1.39	− 0.18	− 0.26	0.40
P value				0.1454	0.3947	0.1939	0.9561	0.6628	0.4647	0.4194	0.6239

gEBVs were represented by the mean ± SD. The difference between two groups was tested by Student's *T* test

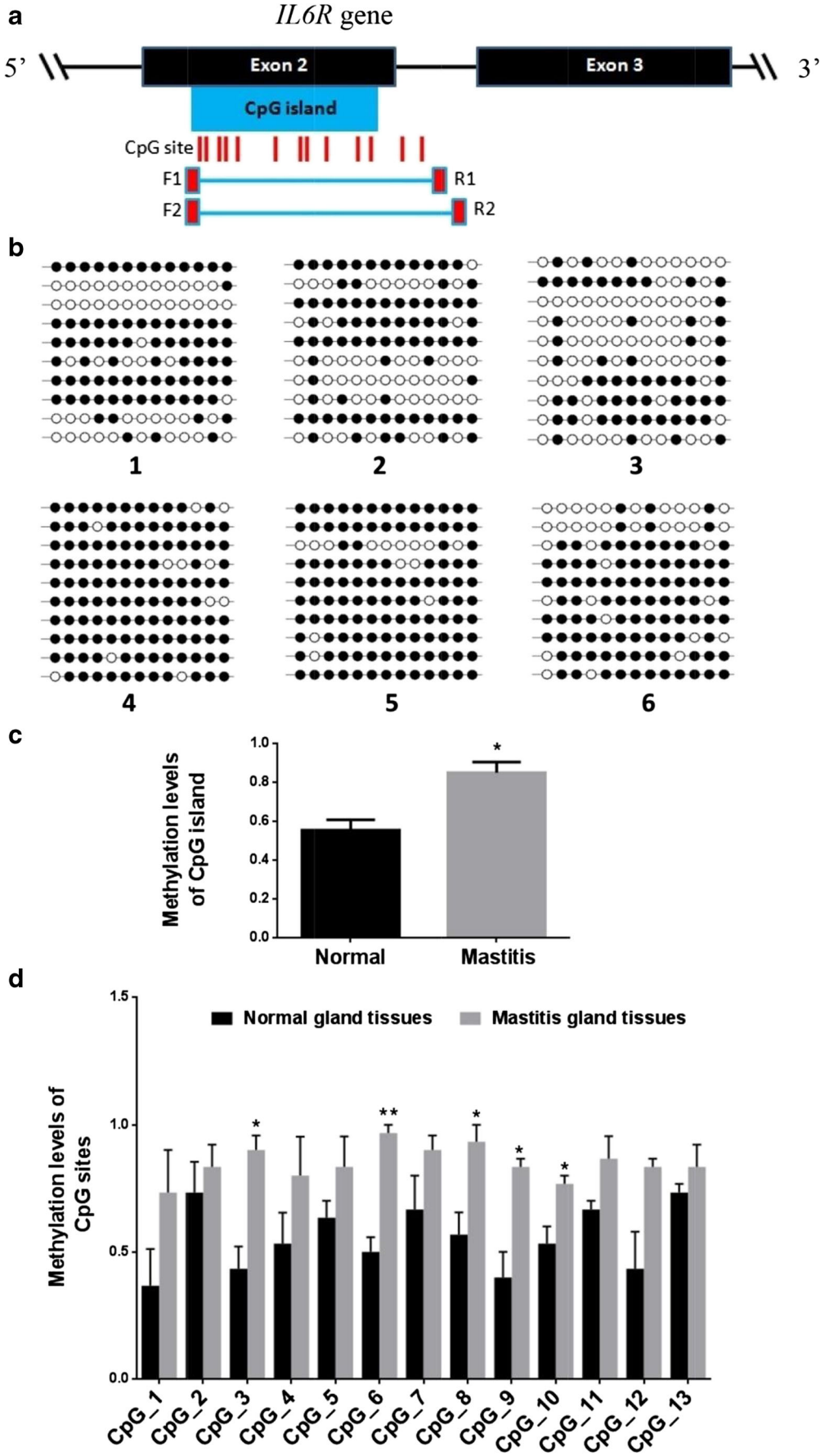
FUA genomic breeding value of fore udder attachment, *RUH* genomic breeding value of the rear udder height, *UC* genomic breeding value of the udder cleft, *UDE* genomic breeding value of udder depth, *FTP* genomic breeding value of front teat placement, *RTP* genomic breeding value of rear teat placement, *FTL* genomic breeding value of teat length

and transfected them separately into MAC-T and HEK293T. Our findings indicated that changes in ESE binding sequences did not enhance the splicing pattern of *IL6R*; hence, the two SNPs did not induce the production of *IL6R-TV.* Moreover, the statistical analysis of sperm samples showed that the two SNPs were not associated with gEBVs of SCS and udder conformation traits. By combining the results of correlation analysis and the exon-trapping system, we concluded that production of aberrantly spliced *IL6R-TV* was not by the two SNPs but through other means.

DNA methylation is an important epigenetic marker that significantly affects natural variation (Heyn et al. 2013). The DNA methylation level in the gene promoter is a powerful epigenetic marker for clinical mastitis (Buitenhuis et al. 2011) and acute mastitis induced by *E. coli (Vanselow et al.* 2006*)*. Similar to that in gene bodies, DNA methylation is positively correlated with gene expression (Yang et al. 2014) and inclusion levels of alternative exons (Maunakea et al. 2013) by affecting the kinetics of transcriptional elongation or by recruiting special splicing factors (Yearim et al. 2015; Fenton et al. 2000; Iannone and Valcárcel 2013). Previous research showed that DNA methylation regulates gene expression, and the present findings indicated that the CpG island was located within alternative exon 2; hence, we speculate that the expression levels of the transcripts of *IL6R* and the inclusion levels of the alternatively spliced exon 2 were regulated by DNA methylation. Our BSP and qRT-PCR results confirmed the hypothesis. Mastitis enriched DNA methylation on the exon 2 of *IL6R*. The high DNA methylation levels upregulated the expression of *IL6R-reference* and *IL6R-TV* and the inclusion levels of exon 2. These results proved that DNA methylation was in correlation with alternative splicing patterns of *IL6R* during inflammatory infection.

Conclusions

We identified one novel *IL6R* transcript that skipped exon 2, and it is upregulated in the mammary gland tissue of clinical mastitis cows. Bovine mastitis increased the DNA methylation level, particularly on some distinct CpG sites, and influenced the expression levels of both inclusion and skipping isoforms and their ratios, resulting in the increased inclusion level of exon 2. DNA methylation rather than SNP regulates the production of an aberrant splice variant of *IL6R* in mastitic cows. The DNA methylation level in exon 2 could be a potential biomarker for monitoring bovine mastitis.


a
IL6R gene
5'
Exon 2
Exon 3
3'
CpG island
CpG site
F1
R1
F2
R2
b
1
2
3
4
5
6
c
Methylation levels of CpG island
1.0
0.8
0.6
0.4
0.2
0.0
*
Normal
Mastitis
d
Normal gland tissues
Mastitis gland tissues
Methylation levels of CpG sites
1.5
1.0
0.5
0.0
*
**
*
*
*
CpG_1
CpG_2
CpG_3
CpG_4
CpG_5
CpG_6
CpG_7
CpG_8
CpG_9
CpG_10
CpG_11
CpG_12
CpG_13

◀ **Fig. 4** Methylation profiles of exon 2 and intron 2 of *IL6R* in mammary tissues. **a** Schematic of the predicted CpG island and the location of the BSP primers. A strong CpG island maps within exon 2 of *IL6R*. The red lines represent 13 CpG sites. **b** Percentage of methylation of mammary gland tissues in normal (1–3) and mastitis-infected (4–6) cows. **c** The frequency of DNA methylation of the predicted CpG island was lower in normal cows (55.40%, $n = 30$ clones) than in mastitis-infected cows (84.83%); asterisk, $P < 0.05$, significantly different from normal controls. Results of BSP of normal (animal #1, 2, and 3) and mastitis-infected (animal #4, 5, and 6) groups. A 233-bp fragment with 13 CpG sites was amplified by using nested BSP. A total of 60 clones (10 for each cow) that had sequences with > 95% cytosine conversion were analyzed through BiQ Analyzer software with the reference *IL6R* gene sequence. A black circle represents methylated CpG sites; a blank circle represents unmethylated CpG sites. **d** Percentage of methylation of the 13 CpG sites of normal and mastitis-infected groups. The methylation levels were higher in the mastitis-infected cows than in normal cows at all the 13 CpG sites, particularly at CpG_3, CpG_6, CpG_8, CpG_9, and CpG_10. One asterisk, $P < 0.05$, significantly different from normal controls. Two asterisks, $P < 0.01$, significantly different from normal controls

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Compliance with ethical standards All experiments were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals published by the Ministry of Science and Technology, China, in 2004. The procedures were approved by the Animal Care and Use Committee of the Dairy Cattle Research Center, Shandong Academy of Agricultural Sciences, Shandong, People's Republic of China.

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