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Single Nucleotide Polymorphism in the Promoter Region of Bovine *Interleukin* 8 Gene and its Association with Milk Production Traits and Somatic Cell Score of Holstein Cattle in Iran

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Background: Interlekin 8 (IL8) is a proinflammatory cytokine and a potent chemotactic and activation factor for neutrophils. It has been reported to be expressed at high levels by various cell types after immune stimulation and mediates neutrophil recruitment in host.
Objectives: The aim of this study was to analyze the relationship between single nucleotide polymorphisms (SNPs) at position -180 G/A in the promoter region of bovine *IL8* gene with milk production traits and somatic cell score (SCS).
Materials and Methods: The part of promoter region in the bovine *IL8* gene containing -180 G/A SNP was screened by a single strand conformation polymorphism (SSCP) and DNA sequencing in Holstein cattle of Iran. The association analysis between different genotypes of IL8-180 SNP and performance traits of Holstein cattle was carried out by Mixed procedure of SAS 9.1 program.
Results: A total of 3 distinct SSCP patterns were observed. Sequence analysis indicated a reported polymorphism at position -180 G/A relative to the start codon. This SNP created a putative binding site for Oct-1 transcription factor which associated with lower SCS. The association of *IL8*-180 genotypes was studied with milk production traits and SCS. The *IL8*-180 associated ($P<0.05$) with milk yield and also tended to associate with SCS ($P=0.06$), but their association with milk fat percentage and milk protein percentage were not significant.
Conclusion: The SNP at position -180 in the promoter region of bovine *IL8* gene was found to be associated with milk yield in Holstein cattle. Gene expression analysis and genome wide association studies were required to confirm the functional role of the *IL8*-180 SNP.

Keywords: Polymorphism; *IL8* gene; Milk production traits; SCS; Holstein cattle

1. Background

Mastitis is a polygenic trait that is caused predominantly by bacterial infection. During mastitis, it is common to observe an increased number of somatic cells (macrophages, neutrophils, and lymphocytes) in milk. These somatic cells play a crucial role in host resistance. In dairy cattle, due to positive genetic correlation between Somatic Cell Count (SCC) and clinical mastitis, most genetic studies focused on milk SCC and clinical mastitis as phenotypic measures to predict the bacterial status of udders (7, 19). Somatic cells migration from the bloodstream to mammary gland tissue occurs as a response to pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1b) and IL8. There is compelling evidence that suggests IL8 is a more powerful neutrophil chemotactic cytokine than the other cytokines (1). Therefore, IL8 is a potential candidate gene for mastitis resistance. The gene encoding bovine IL8 cytokine is mapped on chromosome 6 (6) and contains four exons and three introns, typical of the gene organization of CXC subfamily of chemokines (14). The gene encodes a protein that acts as a potent chemokine for neutrophils when ligating with IL8RA receptor expressed on the surface of neutrophils (21). Identification of SNP in the promoter region of cytokine genes is fundamental for understanding the cytokine gene expression in response to infection diseases. Several studies have confirmed associations between SNPs in the

promoter region of different immune response genes and performance traits in dairy cattle (9, 15, 18).

2. Objectives

The objective of this study was to assess the association between -180 SNPs in the bovine IL8 promoter region with milk production traits and SCS in Holstein cattle of Iran.

3. Materials and Methods

Blood samples were collected from 601 multiprous Holstein dairy cows from one farm at Esfahan province of Iran. Genomic DNA was extracted from EDTA anticoagulated blood using the AccuPrep® (BiONEER, South Korea) genomic DNA extraction kit, according to the manufacturer's instructions.

The 260bp of promoter region (-216 to +44) of the bovine IL8 gene was amplified by polymerase chain reaction (PCR) using the following conditions: Initial denaturing cycle (2 min at 95°C); the amplification step composed of 30 cycles of 95 °C for 30 s, 63°C for 30 s and 72°C for 25 s, and the last step of 72°C for 7 min for final extension. Forward (5'-CAGATGACTCAGATGTGCTCTCA-3') and reverse (5'-CAGGAAAAGCTGCCAAGA-GA-3') primers were designed by Primer3 software (http://frodo.wi.mit.edu/primer3/), according to the promoter region of bovine IL8 sequence (GenBank accession no. AY627308.1). The polymerase chain reaction (PCR) was carried out using a PCR kit (BiONEER, South Korea) with the lyophilized components.

For single-strand conformation polymorphism (SSCP) analyses 2 μL of the PCR product was mixed with 6 μL SSCP gel loading dye (95% formamide, 20 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). Then samples were heated for 5 min at 95ºC and immediately cooled on ice. The total volume was loaded onto a 10% polyacrylamide gel (37.5:1acrylamide/bisacrylamide).

Electrophoresis was carried out at room temperature in 0.5×TBE buffer for 20 h. The gels were subsequently fixed in 10% acetic acid, stained with 0.15% $AgNO_3$ and revealed with 1.5% Na_2CO_3.

For sequence analysis, the PCR products (2 samples) were purified using the QIA quick PCR purification kit (Qiagen, Iran) and bidirectional sequenced by forward and reverse primers. Multiple alignments of the nucleotide sequences for different SSCP patterns were carried out using the CLUSTALW (http:// workbench.sdsc.edu). To evaluate the effect of IL8-180 SNP on transcription factor binding sites within amplified fragment of IL8 promoter region, *In silico* analysis was performed using TFSEARCH v1.3 (www.cbrc.jp/researh/db/ TFSEARCH. html) software.

Statistical Analysis

Test-day records of milk yield, fat percentage, protein percentage and SCC were obtained from the routine milk recoding scheme in the center of animal breeding at Isfahan province. The distribution frequency of SCC values was highly skewed. Therefore, the somatic cell counts were transformed to a log scale and converted to SCS (shooke, 1982).

Frequencies of genotypes, alleles and Hardy-Weinberg test were computed using TFPGA version 1.3. For association studies, the data regarding the milk production traits and SCS were analyzed with the mixed procedure of the SAS 9.1 program according to the following statistical model:

$$y_{ijkl} = \mu + G_i + S_j + L_k + Si_l + e_{ijkl}$$

Where:

y_{ijkl}: phenotypic value of interest traits; μ: mean of the traits; G_i: fixed effect of the IL8 genotype; S_j: fixed effect of season; L_k: fixed effect of lactation; Si_l: random effect of sire and e_{ijkl}: random error.

4. Results

In this study, the amplified fragment of IL8 promoter region revealed three distinct SSCP patterns which were highly reproducible. Different banding patterns were observed, which are indicated by A, B or C band patterns observed were denominated from A to C, as shown in Figure 1.

Sequence analysis of the region revealed a substitution of G to A at position -180 relative to the initiation codon site. Since the forward primer was too close to the -180 SNP, sequencing withthis primer could not distinguishdifferent genotypes of G to Aat IL8-180. Therefore, thesequenc-

ing was performed using reverse primer (Figure 2). According to sequence analysis, theSSCPpatterns of A, B and C is equal to het-erozy-gous (G/A), homozygous (A/A) and homozygous (G/G), respectively for -180 muta-tion. Theobtained sequences for promoter region of thebovine IL8 gene were submitted to NCBI(KF551877, KF551878).

The genotype frequencies of AA, GA and GG for IL-8-180 were 0.270, 0.545 and 0.185 respectively. The estimated frequencies of A and G alleles were 0.54 and 0.46, respectively. The individual frequencies of the genotypes deviated from the Hardy-Weinberg equilibrium for identi-fied SNP by χ^2 test (P= 0.014).

Following a search for possible transcription factor binding sites using TFSEARCH, the ampli-fied sequence of the IL8 promoter region revealed several potential transcription factor binding sites, including AP-1, C/EBPb, NF-kap, c-Rel, GATA-1, Nkx-2 and CdxA. A potential TATA box was also located -114 from start codon site of bovine *IL8* gene. On the other hand, *in silico* analysis of the consequences of identified SNP on potential cis-acting elements predicted that the G to A tran-sition at position -180 does not disrupt any con-sensus sequences for known transcription factors, but creates a putative binding site for Oct-1 tran-scription factor (Table 1) .

The associations between IL8-180 genotypes with milk production traits and SCS are listed in Table 3. Results indicated that different genotypes in this fragment had a significant association with average daily milk yield (P < 0.05). Cows with GG genotype had significantly increased milk yield relative to the GA and AA genotypes. There

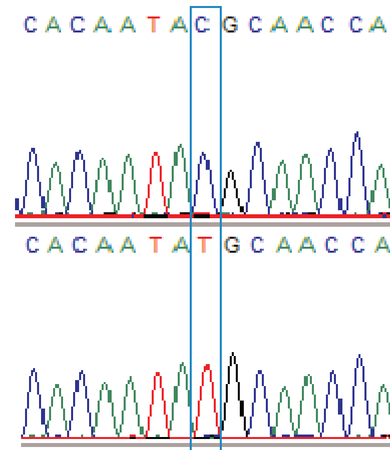

Figure 2. Sequence chromatograph of identified SNP in the promoter region of IL8 gene using reverse primer

was a high tendency to associate between detect-ed genotypes and SCS (P = 0.06), so that the AA genotype had lower SCS rather than AG and GG genotypes. No other significant associations were observed between any of the genotypes at -180 positions for fat percentage and protein percent-age (Table 2).

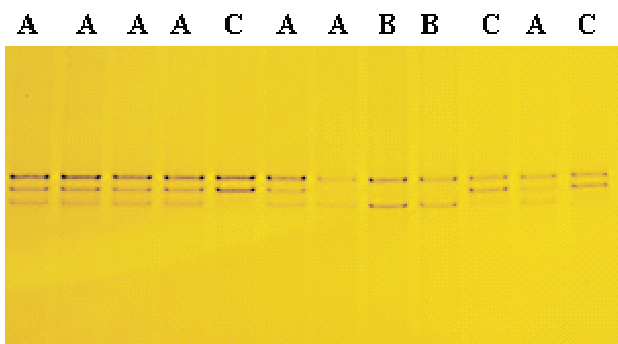

Figure 1. Different SSCP patterns of amplified fragment of bovine IL8 promoter region (A: GA genotype, B: AA genotype and C: GG genotype for IL8-180 SNP)

5. Discussion

The amplified fragment of bovine *IL*8 promoter region revealed 3 SSCP genotypes and sequence analysis of them showed one SNP (G/A) at posi-tion -180. The genotypes of *IL*8 gene significant-ly deviated from the Hardy-Weinberg proportions (P=0.014), indicating that *IL*8 gene is under direc-tional selection. The high frequency of heterozy-gotes (an observed and expected count 327 and 298, respectively) caused this deviation from Hardy-Weinberg equilibrium.

Transcription factors play important roles in the regulation of gene transcription initia-tion. They bind to promoter elements and modulate the relative efficiency of transcrip-tion initiation by activation or repression. The search for transcription factor binding domains revealed that G to A transition at position -180 created a putative binding site for octamer transcription factor-1 (Oct-1). Oct-1 is a transcription factor belonging to the POU family that constitutively expressed in many cell types and specifically interacts with the octamer motif ATGCAAAT (24).

Table 1. Potential transcription factor binding sites, within the promoter region of the bovine IL8 gene

Transcription Factor	Nucleotide Position	Sequence motifs	Wild-type matrix match	Mutated matrix match
AP-1	-214 to -206	atgactcag	98.3	98.3
Oct-1	-188 to -173	gaggttgcgtattgtg	-----	88.6
C/EBPb	-181 to -166	cgtattgtggaattt	90.6	91.2
NF-kap	-174 to -165	tggaatttcc	96.0	96.0
c-Rel	-174 to -165	tggaatttcc	93.4	93.4
GATA-1	-148 to -139	catgatggtg	94.7	94.7
Nkx-2	-138 to -132	cacttgt	90.7	90.7
TATA	-114 to -100	atataaaaagccaca	92.1	92.1
CdxA	-113 to -107	tataaaa	92.9	92.9

Although multiple factors are involved in the control of human IL8 gene transcription (8), the Oct-1 transcriptional factor is particularly important because it can repress the expression of IL8 (2, 10, 26, 29) by displacing the C/EBP transcription enhancer from the IL8 gene promoter (28). Binding of this transcription factor may mediate the reduced sensitivity of promoter sequence of IL8 gene to exogenous and endogenous stimulation (17). In this study, cows with AA genotype at IL8-180 SNP showed significant lower SCS than GG and GA genotypes. The result here suggests that Oct-1 might act as a negative regulatory element for IL8 transcription.

The IL8-180 significantly associated with average daily milk yield, while the associations with fat percentage and protein percentage were not significant. The significant associations were reported with the IL8 mutations and milk yield, 305 day milk protein yield, 305 day corrected milk yield, 305 day milk fat yield, SCS, and milk protein percentage, while their association with milk fat percentage was not significant (3). Leyva-Baca *et al*. (2007) also identified significant associations between the polymorphism of bovine IL8 (A/G at position 2647) with fat yield and udder depth. A possible association between IL8 and fat yield may be due to the IL8 angiogenic properties, including adipocyte movement and adipose tissue metabolism (23).

The association of IL8 promoter region could be explained by the mapping quantitative trait loci (QTL) for milk production traits on bovine chromosome 6. Several studies have investigated segregating QTL with significant effects on milk production traits in the bovine chromosome 6. For example, three QTL affecting milk, fat, and protein production, as well as fat and protein concentration, were found on BTA6 in the Israeli Holstein population (22). According to the confidence interval for QTL location on BTA6, several genes that have some physiological relevance to the milk production traits have been considered primary candidates for the QTL. ABCG2 (5, 13, 20), PPARGC1A (12, 27) and OPN (12) are located in BTA6 that they are potential candidate genes for milk production traits and SCC.

In conclusion, the IL8-180 SNP (G/A transition) that lies in promoter region can be important in the regulation of IL8 transcription. Also, our results provide evidence that the IL8-180 SNP might have potential effects on milk yield and SCS. Therefore, the functional role of this SNP in

Table 2. The associations between IL8-180 genotypes with average daily milk, fat percentage, protein percentage and SCS

					LS means for different genotypes			P for contrast		
Variable	**n**	**mean**	**SD**	***P* value**	**GG**	**GA**	**AA**	**GG vs. GA**	**GG vs. AA**	**GA vs. AA**
Milk yield	541	33.07	6.27	0.04*	36.82	35.05	34.78	0.02*	0.01*	0.66
Fat %	541	3.35	0.52	0.15	3.01	3.10	3.15	0.19	0.05	0.31
Protein %	541	2.94	0.44	0.18	2.70	2.77	2.81	0.21	0.06	0.34
SCS	558	11.15	3.44	0.06	15.51	15.44	14.78	0.84	0.07	0.02*

bovine needs to be analyzed and confirmed by means of gene expression assays. In addition, further studies are needed to confirm the associated effects of the IL8-180 and other polymorphisms in bovine IL8 gene.

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