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Single Strand Conformation Polymorphisms and Sequence Analysis of 5'-Flanking Region of IL-1B Gene in Cattle (Bos taurus)

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ABSTRACT

Interleukin-1β (IL-1β), the prototypical pro-inflammatory cytokine, is produced by macrophages following exposure to bacterial products. Its role is to act upon several cell types at the site of infection to stimulate the production of pro-inflammatory molecules that will cause increase in vascular permeability. Therefore, IL-1β regulates the initiation and development of acute inflammation that may have a role in mammary gland defense during mastitis. Single nucleotide polymorphisms (SNPs) in the 5'-flanking region of this gene can modulate IL-1β function. The aim of the present study was to discover and analyze SNPs in promoter region of IL-1B gene in cattle (Bos taurus). The 5'-flanking region of IL-1B gene was screened by single strand conformation polymorphism (SSCP) in Holstein and Iranian local cattle breeds (50 local and 50 Holstein). A total of 4 distinct SSCP patterns were observed, which further revealed 5 novel SNPs upon sequence analysis in Iranian local breed. From the SNPs identified in this region, polymorphism at nucleotide position -534 was found to lie in the vicinity of potential GATA and ZNF transcription factor binding sites. The SNPs identified at -383 position was shown to be present within the putative ETS factor and also core sequence of CARE transcription factor. Two SNPs at positions -534 and -340 were found within the EBF binding site. The SNPs identified in the 5'-flanking region of IL-1B gene may serve as potential candidate genetic marker(s) for disease resistant traits in cattle.

Keywords: 5' flanking region, IL-1B, SNPs, Transcription factors.

INTRODUCTION

There is great need for an approach at the DNA level that deals with dairy cattle selection for disease resistance. This method will allow genetic evaluation of the animals and will enable breeders to more accurately select those animals that not only express the desirable traits phenotypically, but also those that show favorable underlying genetic criteria. This has mostly been achieved by marker-assisted selection. Diseases resistance is a complex characteristic influenced by genetic component and environmental factors, including infection pressure. It has been observed that several diseases affecting cattle have an inflammatory component mediated by cytokines (Mrak and Griffin, 2001), which are regulatory proteins, such as the interleukins and lymphokines, that are released by cells of the immune system and act as intercellular mediators in the generation of an immune response.

Currently, dairy cattle research has been focused on the role of cytokines in bacterial infections, such as Mastitis, which have a significant influence on the dairy economy worldwide. In this regard, interleukin-1 (IL-1), interleukin-8 (IL-8) and tumor necrosis factor-a (TNF- α) are known to be important inflammatory mediators. They are involved in the initiation and development of acute

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inflammation (Tracy, 1994). The IL-1 is a primary inflammatory cytokine and has been implicated in mediating both acute and chronic pathologic inflammatory diseases. This cytokine plays an important role in host resistance against intracellular microorganisms.

There are ten individual members of the IL-1 gene family, of which the four primary members are IL-1A, IL-1B, IL-18 and IL-1 receptor antagonist (IL-1Ra). IL-1A, IL-1B and IL-18 are agonists while IL-1Ra is the specific receptor antagonist for IL-1A and IL-1B, but not for IL-18 (Dinarello, 2009). It is reasonable to speculate that genetic variations affecting expression activities of members of the IL-1 gene cluster represent genetic risk factors for susceptibility and outcome in inflammatory diseases (Nicklin *et al*., 1994). The 5'-regulatory region contains constitutive promoter elements (such as transcription factor sites), enhancers, repressors, the determinant of tissue-specific gene expression, and other responsive elements (Cooper, 1992), therefore, sequence variations can be important. Several studies indicate that variations in cytokine expression are associated with disease activity in immune-mediated or inflammatory disorders (Mosmann and Sad, 1996; Hansen *et al*., 2004). In human, several polymorphisms within the 5'-flanking region of the IL-1 genes cluster have been reported to influence the transcription of IL-1, including polymorphisms within the IL1A gene (-889) C/T, and the IL-1B gene (-511) C/T, (-31) C/T. The IL-1B (-511) C/T has been associated with either susceptibility or outcome in meningococcal disease in the British population (Read *et al*., 2003). In another study, a polymorphism of the IL-1B gene (a C/T transition at position -511) was reported to be associated with coronary artery disease (Acoviello *et al*., 1999).

To date, on the basis of current knowledge, there have been no reports on nucleotide polymorphisms from the 5'-flanking region of the IL-1B gene in cattle. Hence, our objective was to study sequence variations and analysis in the 5' -flanking region of the bovine IL-1B gene.

MATERIALS AND METHODS

Blood samples were collected from the individual local and Holstein cattle breeds. In the local breed, samples were collected in a village of Kohgiluyeh and Boyerahmad province, while the samples of the Holstein breed were taken from one single farm (Isfahan Milk and Meat farm). AccuPrep® genomic DNA extraction kit was used to extract genomic DNA from 2 ml EDTA–anticoagulated peripheral blood samples (50 Local, 50 Holstein). Using Primer3 software (http://frodo.wi.mit.edu/primer3/), two new primer pairs were designed from IL-1B sequence (GenBank accession no.AF026543.1) to amplify 5'-flanking region of the gene (Table 1). Twenty five µl of polymerase chain reaction (PCR) mixture were carried out in 0.2 ml PCR tubes, using a PCR kit with the lyophilized components. Each tube contained 1.5 units of Taq DNA polymerase, 10 mM of Tris-HCl (pH 9), 50 mM of KCl, 1.5 mM of $MgCl_2$ and 200 µM of each dNTP. The following PCR conditions were used: denaturation at 94°C for 5 minutes; 35 amplification cycles of denaturation at 94°C for 45 seconds, annealing at 63ºC (62ºC for IL1B-F2) for 45 seconds, and extension at 72°C for 30

Table 1. The features of primers used to amplify 5'-flanking regions of IL-1B gene.

Fragment	Primer sequence	Fragment size (bp)	Fragment location	Annealing temperature (°C)
IL1B-F1	*5′-ggatgagcttgtctccaagg-3′* *5′-accaaaatagcgggttttcc-3′*	349	-629 to -280	63
IL1B-F2	*5′-ggaaaacccgctattttggt-3′* *5′-atgaaggttggctggagaga-3′*	338	-300 to +38	62

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seconds; a final 7 minutes extension at 72°C. For single-strand conformation polymorphism (SSCP) analysis, 3 µl of the PCR product was mixed with 6 µl stop solution (95% formamide, 20 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). The samples were heated for 10 minutes at 95ºC and immediately cooled on ice. The total volume was loaded onto a 10% polyacrylamide gel (37.5:1 acrylamide/bisacrylamide). Electrophoresis was carried out at room temperature in 0.5_TBE buffer for 24 hours. 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, purified PCR products were commercially sequenced with both forward and reverse primers. The multiple alignments of the nucleotide sequences of different SSCP patterns were carried out using the CLUSTALW (http:// workbench.sdsc.edu). The sequences for 5' flanking region of the IL-1B gene were submitted to NCBI (JF450743, JF450744, JF450745, JF450746, JF450747, JF450748 and JF450749). The putative transcription factor binding sites within 5' flanking region were searched by MatInspector professional 8.2 from Genomatix Software Complex (http://www.genomatix.de) at the default.

RESULTS

In this study, we described through PCR-SSCP and DNA sequencing the single nucleotide polymorphisms (SNPs) within the 5' flanking region of the IL-1B gene in Iranian local and Holstein cattle breeds. The 100 animals analyzed revealed four PCR-SSCP patterns for IL1B-F1 fragment only in the local breed, but other fragments showed single SSCP pattern in both breeds. The individual SSCP patterns were highly reproducible. The different band patterns observed were denominated from A to D, as shown in Figure 1. Variant C was the only variant identified in Holstein breed samples. Variant A was a rare variant found only in two individuals of the local breed among the investigated samples.

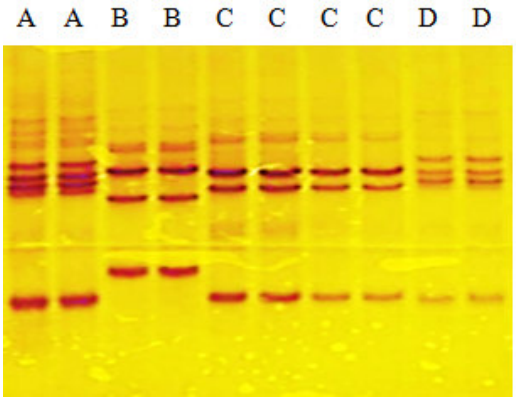

Figure 1. Different SSCP patterns of IL1B-F1 fragment.

The frequencies of SSCP patterns for IL1B-F1 fragment in the local breed were obtained by direct counting. The frequencies of A, B, C, and D patterns for IL1B-F1 fragment were 0.04, 0.28, 0.52 and 0.16, respectively. DNA sequences of PCR-SSCP patterns of IL1B-F1 fragment showed that the C pattern matched the sequence AF026543.1 previously submitted to GenBank, while the A, B and D, SSCP variants corresponded to new DNA sequences JF450743 through JF450748. The nucleotide sequencing of these SSCP patterns revealed single nucleotide polymorphisms (four transitions, one transversion) at five positions: a T to C substitution at position −591, C to T substitution at position −534, C to T substitution at position −383, C to G substitution at position −357 and an G to A substitution at position −340 (Figure 2). Following a search for possible transcription factor binding domains using MatInspector, a total of 77 different putative sites were identified, 29 of which had 100% core similarity match (Core similarity score= 1) and more than 90% matrix similarity match (Matrix similarity score= 0.9).The search for transcription factor binding domains revealed that some of them coincided with SNP positions (Table 2).

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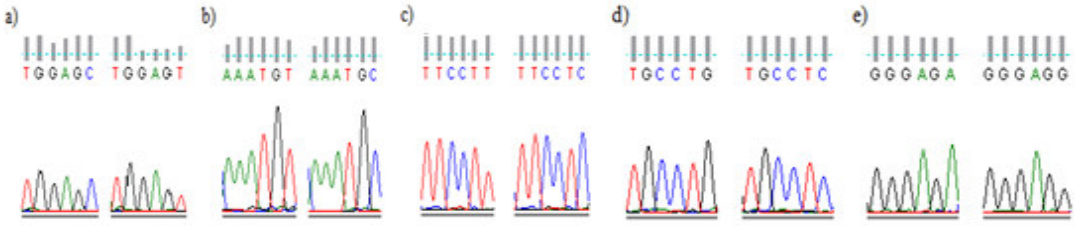

Figure 2. Sequence chromatographs of SNPs identified in the 5' flanking region of IL-1B gene at positions -591(a), -534(b), -383(c), -357(d) and -340(d).

DISCUSSION

It is clear that genetic factors play an important role in determining risk of diseases in cattle, and many DNA polymorphisms associated with disease resistance in various genes have been reported. In other words, the drastic role of cytokines in host defense during infectious and inflammatory diseases have been well established. Among others, IL-1, IL-8 and TNF-α are known to be important inflammatory mediators. They are involved in the initiation and development of acute inflammation. In this study, we characterized the 5'-flanking region of bovine IL-1B gene by PCR-SSCP and DNA sequencing of 667bp length and performed polymorphism analysis in Iranian local cattle and Holstein breeds. Considerable variation was found to exist in this region as revealed by 5 different SSCP variants which further identified 5 single nucleotide polymorphic sites (SNPs).

Holstein dairy cattle appeared monomorphic in this respect as only one IL1B-F1 genotype (SSCP pattern of C) was found in these animals. Generally, breeding objectives in dairy cattle have focused almost exclusively on milk production traits such as milk, fat, and protein yield. The result is a significant reduction in the genetic potential of dairy cattle to disease resistance. In contrast to local samples, absence of genetic variation in Holstein samples showed that selection for milk production traits decreased variation in the gene affecting immune system within a population. Transcription factors play important roles in determining spatial and temporal gene expression patterns. They can directly regulate gene transcription by interacting with cis-regulatory DNA elements in specific genes. The search for transcription factor binding domains revealed the presence of many putative sites within IL1B-F1 fragment (Figure 3), few of which coincided with SNP positions (Table 2). One polymorphism at position -534 (T-C transition) lies neighbor to a GATA binding factor site. No information has yet been published about the presence and the possible role of GATA-sites in IL-1B gene.

Table 2. Transcription factors within 5'-flanking region of IL-1B gene which coincided with some of the identified SNPs.

Transcription factor	Nucleotide position	Strand	Core match	Matrix match	Sequence motif[a]
GATA	-548 to -535	+	1.000	0.923	*gcaAGATcaaatg*
ZNF	-545 to -521	+	1.000	0.994	*agatcaaatgtctCCCCagaggc*
EBF	-538 to -515	+	1.000	0.945	*aatgtcTCCCcagaggcttcagt*
ETS	-391 to -370	-	1.000	0.951	*gagcatagGGAAgtgacagta*
CARE	-388 to -377	-	1.000	0.917	*acacaGAGGca*
EBF	-344 to -321	+	1.000	0.926	*actttgTCCCcggagatccgaaa*

[a] Nucleotide in upper case indicate the core sequence.

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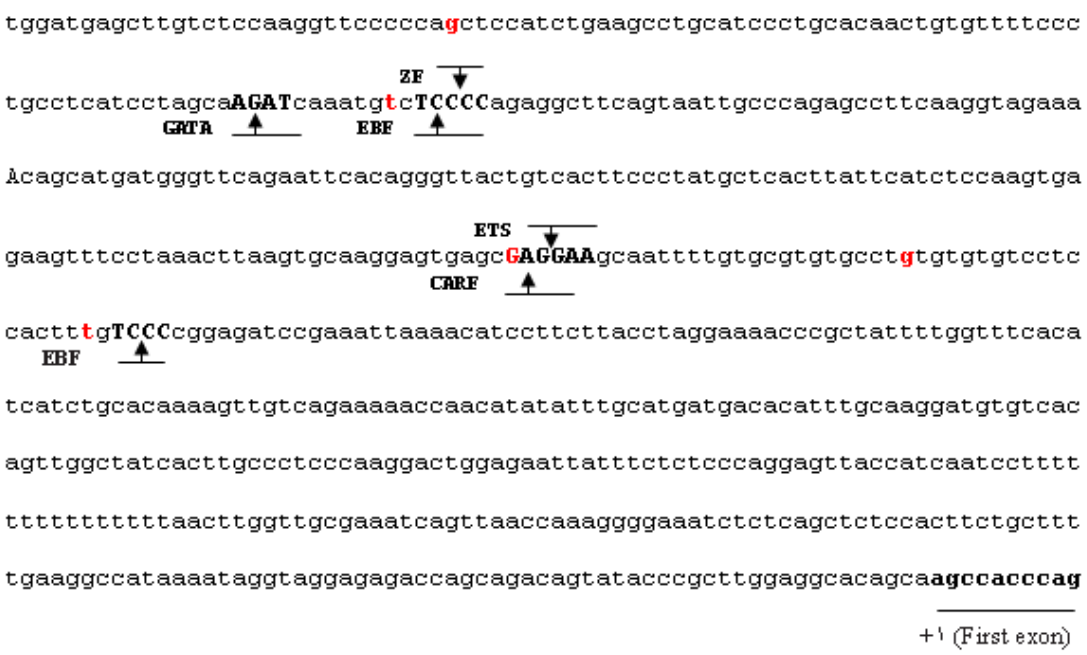

Figure 3. Putative transcription factor sites (Bold font) and SNPs (red color) in bovine 5′ flanking re of IL-1B gene.

The GATA family of transcription factors are zinc finger proteins that bind to the consensus sequence (A/T)GATA(A/ G) via a DNA-binding domain (Charron *et al*., 1999) containing two zinc fingers to activate target genes. The GATA proteins play important roles in cell differentiation and homeostasis in all eukaryotes. Yu *et al*. (2002) demonstrated that mutation of GATA motif downstream of the major transcription start site of the E4bp4 (adenovirus E4 promoter-binding protein) gene impaired not only the basal promoter activity but also the IL-3 inducibility of this gene. The E4BP4 protein was initially identified by its ability to recognize and repress the adenovirus E4 promoter (Cowell and Hurst, 1994) and was subsequently identified as NF-IL3A in T cells to be capable of binding and activating the human IL-3 promoter (Zhang *et al*., 1995). In sheep, significant difference was not found in IL-2 transcription between the genotypes of single nucleotide (G/C) at GATA-3 factor (Luhken *et al*., 2005).

This single nucleotide polymorphism is located within a potential C2H2 zinc finger transcription factor2 (ZNF) binding site. The C2H2-type zinc finger proteins represent a large and important transcription factor family in eukaryotic (Laity *et al*., 2001).Up to now, at least 20 different classes of zinc finger motifs have been identified. The most clearly identified zinc finger motif is C2H2 type zinc finger, a lot of which can bind to specific DNA sequences (Wang *et al*., 2008a). A potential C2H2 zinc finger binding site was found in the promoter region of the human IL-2RB gene (Hongling *et al*., 2008). Wang *et al*. (2008b) reported that zinc finger protein Zbtb7b acted in peripheral of $CD4^+$ T cells to suppress CD8-lineage gene expression, including that of CD8 and cytotoxic effector genes perforin and Granzyme B, and was important for the proper repression of interferon-g (IFN- g) during effector differentiation.

Another interesting polymorphism was at position −383, where a C to G transition was observed. A putative ETS was found to be present in the region −370 to −391

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bp upstream to 5′-flanking region of bovine IL-1B gene. The Ets family of transcription factors has been implicated in regulation of cellular in various cell types such as proliferation, differentiation, immune responses, and apoptosis. The Ets transcription factors are characterized by the conserved Ets-domain, which mediates binding to purine-rich *5-GGAA/T-3* core consensus sequence (Sharrocks, 2001). Individual Ets proteins show preferences to specific flanking sequences around the core binding sequence, which contributes to regulation of specificity in responses (Graves and Petersen, 1998). The Ets transcription factor family members Ets1, Ets-2, and ESE-1 have positive effect on transcription of Auto-immune regulator (AIRE) gene. Site-directed mutagenesis and transfection studies revealed that two of the three Ets binding sites in AIRE promoter are functional and this finding has been confirmed by the electrophoretic mobility shift assay (Murumagi *et al*., 2006). The presence of this putative transcription factor binding sequence in the IL-1B promoter coupled with the immunity nature of the IL-1B may suggest a possible basis for an increase in IL-1β production when the immune system is stimulated. This SNP was found to be present in the core sequence of putative recognition sequence for CAER (Calcium response element) transcription factor. Calcium Response Factor (CaRF) was first identified as a transcription factor based on its affinity for a neuronal-selective calcium-response element (CaRE1) in the gene encoding Brain-Derived Factor (BDNF). DNA binding domain of CaRF has been highly conserved during evolution and CaRF binds DNA directly in a sequence-specific manner in the absence of other eukaryotic cofactors (Pfenning *et al*., 2010).

Two SNPs at positions -534 and -340 were found within the Neuron-specific olfactory factor (Early B-cell, EBF) binding site. The early B-cell factors are evolutionarily conserved transcription factors with specific roles in the and maturation of several cell lineages including B-progenitor lymphoblasts (Hagman and Lukin, 2005) neuronal precursors (Garel *et al*., 1997) and osteoblast progenitors (Kieslinger *et al*., 2005). Inactivation of EBF genes blocks normal development pathways, resulting in the accumulation and of progenitor cells, leading to tumorigenesis (Zardo *et al*., 2002).

In conclusion, to our knowledge, this is the first study of SNPs identification in the upstream non-coding region of the bovine IL-1B gene that could up-regulate gene expression. The functional role of these identified polymorphic sites in bovine needs to be analyzed and confirmed using gene expression analysis.

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تفاوت فرم فضایی رشته های منفرد و تجزیه و تحلیل ناحیه ٔ۵ کناری ژن اینترلوکین ۱- بی (IL-1B) در گاو (Bos taurus)

م. محقق دولت آبادی. ، ج. حبیبی زاد. ، و م. قادری زفره ای

چکیده

اینرلوکین ۱ بتا، سیتوکین جزئ اصلی پیش التهابی، با قرار گرفتن ماکروفاژها در معرض محصولات باکتریایی تولید می شود. این سیتوکین با تاثیر بر محل آلودگی در انواع سلول های مختلف تولید مولکول های پیش التهابی را تحریک کرده و منجر به افزایش نفوذپذیری عروقی می شود. از این رو، اینتر لوکین ۱ بتا شروع و توسعه التهاب حاد را تنظیم می کند. چند شکلی تک نوکلئوتیدی در ناحیه ٔ۵ کناری این ژن می تواند نقش و فعالیت اینترلوکین ۱ بتا را تعدیل کند. هدف از این مطالعه کشف و تحلیل چند شکلی های تک نوکلئوتیدی در ناحیه ٔ۵ کناری این ژن در گاو بوده است. برای این منظور، این ناحیه از ژن اینترلوکین توسط تکنیک تفاوت فرم فضایی رشته های منفرد (SSCPs) در گاوهای دو نژاد هلشتاین و بومی غربال گری شد. در کل ۴ الگوی (SSCPs) مجزا مشاهده شده که با تعیین توالی نوکلوتیدی آنها تعداد ۵ چند شکلی تک نوکلئوتیدی جدید در نژاد بومی شناسایی شد. از این چند شکلی های تک نوکلوتیدی شناسایی شده در این ناحیه، چند شکلی جایگاه ۵۳۴- در نزدیکی جایگاه های۲ فاکتور رونویسی GATA و ZNF قرار داشتند. چند شکلی تک نوکلئوتیدی جایگاه ۳۸۳- در داخل فاکتور رونویسی ETS و همچنین توالی هسته فاکتور رونویسی CARE مشاهده شد. چند شکلی های تک نوکلئوتیدی ۵۳۴- و ۳۴۰- در حوزه جایگاه فاکتورEBF یافت شدند. چند شکلی های تک نوکلوتیدی شناسایی شده در ناحیه ٔ۵ ژن اینترلوکین ۱- بی ممکن است بعنوان نشانگرهای ژنتیکی جهت ویژگی های مقاومت به بیماری در گاو بکار گرفته شوند.

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