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Brief Report

Sequence Characterization in 3′-Flanking Region of Bovine *TNF-α*: Association with Milk Production Traits and Somatic Cell Score in Holstein Cattle of Iran

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Background: Tumor necrosis factor- α (TNF-α) is a cytokine that was identified as a factor with a wide range of proinflammatory activities. The expression of bovine *TNF-α* in mammary tissue during pregnancy seems to have a role in development of the corresponding glands.
Objective: Single nucleotide polymorphisms (SNPs) were defined in 3′-flanking region of bovine *TNF-α* in cattle. Moreover, its association with performance traits in Holstein dairy cattle was evaluated.
Materials and Methods: The 3′-flanking region of *TNF-α* was screened by single strand conformation polymorphism (SSCP) and DNA sequencing in Holstein cattle breed. SAS statistical software was used to analyze the relationship between different genotypes of amplified fragment with milk production traits (daily milk yield, fat and protein percentage) and somatic cell score (SCS).
Results: A total of 6 distinct SSCP patterns were observed. It was further revealed to be 3 novel SNPs. Statistical analysis revealed that different haplotypes of amplified fragment in the *TNF-α* 3′-region had a significant effect on average daily milk production ($p < 0.05$), but no such correlation was established with fat percentage, protein percentage and SCS.
Conclusion: The association identified in the 3′-flanking region of *TNF-α* may have potential to serve as candidate genetic marker for genomic selection in dairy cattle.
Keywords: Milk production traits, SCS , SNPs , *TNF-α,* 3′-flanking region

1. Background

The breeding programs on livestock have been largely focused for the dairy and meat related traits with little attention to health characteristics, such as immune responses. Thus, breeding strategy considering both the common traits and the characteristics that improve animals' resistance to varieties of diseases promise a greater potential for years to come (1). For the latter, *i.e.*, pathogen resistance, taking a gene candidate approach can help to speed up the screening procedure through development of new molecular markers. Amongst the candidate genes, cytokines and their receptors stand out as prominent genetic markers to screen for disease resistance. The reason is the fact that cytokines play a pivotal role in mobilizing inflammatory cells in response to infectious challenges (2). For example, it is common to observe an increased number of somatic cells (macrophages, neutrophils, and lymphocytes) in milk of dairy cattle during mastitis. Therefore, to predict the bacterial status of mammary gland, most genetic studies focused on milk somatic cell count (SCC) and clinical mastitis as phenotypic markers (3).

The identification of large number of cytokine genes and disclosure of their mechanism has opened innovative frontiers in disease diagnostics and therapy. Genetic variation in cytokine genes associated with innate resistance is likely to contribute to mastitis susceptibility in dairy cattle. This has been demonstrated with the variants of *chemokine receptor gene 1* (CXCR1) (4-5), CXCR2 (4, 6) and the genes

encoding for several cytokines, including *TNF-α* (7-8), *IL-8* (9) in Holstein dairy cows. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokines that is being produced by different types of immune cells. It is especially causes the activation of macrophages during inflammatory process (10-11). TNF-α stimulates the proliferation, differentiation and activity of many immune system cells (12).

The bovine *TNF-α* contains four exons and three introns on chromosome 23 (13). Reports are indicative of single nucleotide polymorphisms (SNPs) in the *TNF-α*. Further conclusive studies are suggestive the association of *TNF-α* with performance traits and immune function in cattle (14-17).

2. Objectives

Information regarding genetic variation within 3′-flanking region of the bovine *TNF-α* in missing from the literatures. Therefore, it was planned to seek for such genetic variation and establish the correlation of the variation with milk production traits and SCC in Holstein cattle, if there is any at all.

3. Materials and Methods

Total genomic DNA was isolated from 200 EDTA treated blood samples of Holstein dairy cows by AccuPrep® genomic DNA extraction kit. Forward primer (5′- GGCTCCAAGCATCCAACTT-3′) and reverse primer (5′- GGCTTCCCACCTCAG-3′) were designed by primer 3 (http://frodo.wi.mit.edu/primer3/) according to bovine *TNF-α* sequence (GenBank: AF011926.1). A fragment of 305 bp from 3′ region (3259 to 3563) was amplified in 25 µL. The PCR products were diluted in a denaturing solution, denature at 94 °C for 5 min, rapidly cooled on ice and resolved on 11% polyacrylamide gel at 25 ºC, 150 V for 16 h. Then, the gel was silver-stained. The PCR products that revealed different SSCP pattern were selected for DNA sequencing. The multiple alignments of the nucleotide sequences were carried out for different SSCP patterns using the CLUSTALW (http://workbench.sdsc.edu).

3.1. Statistical Analysis

The genotype frequencies of amplified fragment of *TNF-α* were estimated by direct counting. Data for milk production traits including average daily milk yield, fat percentage, protein percentage and somatic cell count were obtained from the farm records. The statistical software SAS (Mixed procedure) was used to analyze the relationship between different SSCP patterns with milk production traits and somatic cell counts by means of least-square method. The association analysis was calculated according to the following statistical linear model:

$$Y_{ijklm} = \mu + G_i + L_j + F_k + S_l + C_m + e_{ijklm}$$

Where: Yijklm: Phenotypic value of the milk production traits and somatic cell count of the animal k with a genotype i, µ: overall mean, Gi: the fixed effect of the *TNF-α* genotype, Lj: the fixed effect of lactation, Fk: the fixed effect of season, S_l: the random effect of sire, Cm: the fixed effect of calving age and e_{ijklm}: random error. The distribution frequency of SCC values was highly skewed; therefore, the values of SCC were transformed to the logarithm scale and converted to somatic cell score (SCS).

4. Results

A 305 bp fragment of 3′-flanking region of *TNF-α* was successfully amplified in all samples. PCR-SSCP was performed to detect genetic variation that might be present. Then, the amplified region of bovine *TNF-α* revealed 6 distinct SSCP patterns (A, B, C, D, E and F) in animal analyzed (**Fig. 1**) which their genotype frequencies were 0.49, 0.19, 0.18, 0.13, 0.05 and 0.05, respectively. Due to low frequencies, the E and F SSCP patterns were excluded from association analysis. Sequence analysis of amplified fragment revealed three novel SNPs (two substitutions and one insertion): a G to T substitution at position 3388, a G to A substitution at position 3431 and an insertion of A at position 3399 (**Fig. 2**).

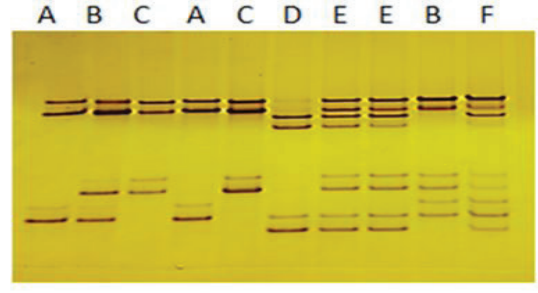

Figure 1. Different SSCP patterns of amplified fragment at the 3'-flanking region of the bovine *TNF-α*.

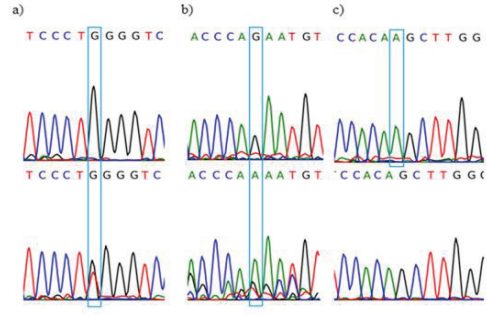

Figure 2. Sequence chromatograph of identified SNPs in the 3'-flanking region of the bovine TNF-α gene. a) substitution of G to T (heterozygous condition), b) substitution of G to A and c) Insertion of A at position 3399.

Table 1. Estimates of effects associated with different haplotypes in the 3'-flanking region of the bovine TNF-α on average milk yield, fatand protein percentages, and SCS.

				LS means for different SSCP patterns			
Variables	**Mean**	**p-value**	**SD**	**A**	**B**	**C**	**D**
Milk Yield	33.34	0.04*	5.89	33.65	34.15	33.38	31.80
Fat %	3.36	0.15	0.46	3.35	3.38	3.33	3.47
Protein %	2.93	0.70	0.32	2.93	2.92	2.91	2.96
Somatic Cell Score	1.49	0.46	0.94	1.46	1.56	1.50	1.32

Note: LS: least square, SSCP: single strand conformation polymorphism, SD: standard deviation, *: significant.

The statistical analysis of *TNF-α* genotypes effect on milk production traits (average daily milk, fat and protein percentage) and somatic cell score showed significant differences for average milk yield ($p < 0.05$) .The higher milk yield was characterized for group of cows with the B haplotype while the D haplotype was unfavorable for milk yield, because it had the lowest value. The identified haplotypes displayed no significant association with fat percentage, protein percentage and SCS (**Table 1**).

5. Discussion

Improvement of milk production traits and increase of genetic resistance to disease are the main objectives of dairy cattle breeding program. This progress can be achieved with traditional selection programs along with genetic markers. Marker assisted selection (MAS) in dairy cattle apply quantitative trait loci (QTL) and candidate genes, which affect phenotype of cattle. The genetic progress analyses using marker assisted selection can provide genetic evaluation more accurately and in a time efficient manner, which is needed for realization in cattle breeding.

Immune genes like *TNF-α* which are being expressed in mammary tissue during pregnancy, appear to contribute in development of mammary gland (15, 18-20). Hence, the bovine *TNF-α* gene might be a strong candidate for mammary gland health and development. In this study, we characterized the part of 3′-flanking region of the bovine *TNF-α* gene by PCR-SSCP and DNA sequencing in dairy Holstein cattle. Considerable variation was found to exist in this region as revealed by 6 different SSCP variants which further identified 3 novel SNPs. To date, on the basis of current knowledge, our results is the first report on nucleotide polymorphisms detection in the 3′-flanking region of the bovine *TNF-α* gene in Holstein cattle. In the dbSNP database, there are 28 SNPs in the bovine *TNF-α* gene, but validation status for most of them is unknown (21). In the other hand, sequence analysis of 5′-flanking region in the bovine *TNF-α* revealed three SNPs that were heritable according to the expected manners (22).

Due to its biological functions, the bovine *TNF-α* has been proposed as a potential genetic marker for immunity to mastitis. The gene is located in the chromosome regions which has been shown to contain QTLs contain loci linked to susceptibility to udder inflammation (14, 23-24). In addition, *TNF-α* genotypes were associated with a lower number of mastitis cases in lower parities and a higher number of mastitis cases in higher parities of dairy cattle (7). However, in this study, no significant association was found between *TNF-α* genotypes and SCS.

The genotypes of amplified fragment in the 3′-region of *TNF-α* significantly associated with average daily milk yield ($p < 0.05$), but their associations with fat percentage, protein percentage were not significant. In several studies, significant associations were reported with the *TNF-α* SNPs and performance traits in dairy cattle. For example, milk yield and milk fat yield were significantly different for the *TNF-α* -824A/G gene polymorphism in Black Pied cattle (25). In addition, Kawasaki *et al*. (2014) reported that SNPs in the promoter region and exon of *TNF-α* were associated with reproductive performance in cattle. Furthermore, their findings provided evidence that the migration, gene expression and production of cytokines in immune cells were different based on the *TNF-α* promoter genotype (17). Significant difference was found between genotypes of the bovine *TNF-α* at promoter region (824A/G) with milk yield and milking rate in Red Steppe cattle breed (26).

6. Conclusion

The genes associated with milk production and udder health can be evaluated as a genetic markers candidate for quantitative traits in dairy cattle. The results of our study confirmed that *TNF-α* genotypes may cause differences in milk production. Therefore, the *TNF-α* gene could contribute to the progress in dairy cattle.

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