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Evaluation of Lactoferrin, *CD74*, *GLYCAM1*, and *FCER1G* Genes Expression on Mammary Epithelial Tissue between Holstein Cows with Clinical Mastitis and Healthy Cows

Research Article

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ABSTRACT

Mastitis is one of the most economically significant diseases in the dairy cattle industry, which increases treatment costs and reduces milk production, genetic advancement of the herd, and reduced milk quality. In the present study, the expression level of some influential genes involved in the immune system as well as disease including lactoferrin, CD74, glycosylation dependent cell adhesion molecule 1, and *FCER1G* genes were investigated. For this purpose, the breast tissue was separated from 10 cows with clinical mastitis and 10 healthy cows and total RNA was extracted from epithelial cells of mammary tissue of healthy and diseased cows using a kit. Then cDNA was synthesized using kits and the relative expression of the genes was measured using the real-time PCR technique. The 18S rRNA gene was used as a housekeeping gene. The relative expression of genes was measured by the $2^{-\Delta\Delta ct}$ method and statistical differences between healthy and patient groups were examined by t-test. The results showed that the expression levels of *Lf*, *CD74*, and *FCER1G* genes in the group of cows with clinical mastitis were 2.5 ($P<0.0003$), 6 ($P<0.0004$), and 2 ($P<0.002$) times higher than healthy cows, respectively. In contrast, the expression level of the *GLYCAM1* gene was significantly lower (4 times) in the group of cows with clinical mastitis comparing healthy cows ($P<0.0074$). Given the important role of these genes in immune responses, it is important to study them as candidate genes for resistance to mastitis disease.

KEY WORDS *CD74* gene, *FCER1G* gene, *GLYCAM1* gene, *Lf* gene, mastitis.

INTRODUCTION

Mastitis is the most common and costly disease in dairy cattle (Abebe *et al*. 2016). It is an inflammation of the mammary gland which causes physical, chemical, and microbial changes in the milk, as well as changes in the tissue of the mammary gland (Pawlik *et al*. 2014). Mastitis can be occurred by more than 100 different microorganisms (Huang *et al*. 2011). The disease occurs in both clinical and subclinical forms. In both cases assessment of mastitis is quantitatively done using somatic cell count (SCC), as there is a positive correlation between mastitis infection severity with SCC with a predominance of neutrophils, macrophages, and lymphocytes (Peters *et al*. 2015). During this inflammatory reaction, leukocytes invade the milk from the blood because the cow's immune system responds to the bacterial infection, thus increasing the concentration of somatic cells in the milk (Hemati *et al*. 2014). Subclinical mastitis is characterized by the absence of external signs, but in clinical mastitis can be diagnosed by signs such as heat, swelling and discoloration of the udder, abnormal secretion, and systemic reactions, such as fever and loss of

appetite (Peters *et al.* 2015). Lactoferrin (Lf) is a member of the transferrin family and is found in milk, bile, saliva, and a variety of biological fluids. Lactoferrin has many biological functions including antibacterial, antiviral, and antitumor activities as well as immunomodulatory properties. The bovine Lf (bLf) concentration increases during mammary infection. Accordingly, bLf has been considered an important component in mammary gland innate defense mechanisms against mastitis (Pawlik *et al*. 2009; Huang *et al*. 2011).

The *bLf* gene is located on the long arm of chromosome 22 in the bovines genome spanning about 34.5 Kb of genomic DNA (Teng, 2002). In bovine milk with subclinical mastitis, the number of somatic cells was positively associated with Lf levels. Therefore, the Lf gene has been introduced as a candidate gene for resistance to mastitis. Expression of Lf is rather low in the lactating gland but increases markedly during mammary gland infection. This implies that there is a possible relationship between the bLf gene and mastitis in dairy cattle (Chopra *et al.* 2015). The *GLYCAM1* gene is a member of the glycoprotein mucin family and a component of the milk fat globule membrane (Le Provost *et al*. 2003). It is an endothelial glycoprotein that is secreted by the lymph nodes and acts as a ligand for the surface of leukocytes L-selectin to mediate lymphocyte extravasation. GLYCAM1, which is present in the mammary gland, may have different functions from the type found in the lymph nodes because the form in the mammary gland is non-sulfated and cannot interfere with L-selectin (Dowbenko *et al*. 1993). GLYCAM1 in the mammary glands may play a protective role in cellular transport and secretion of milk fats (Le Provost *et al*. 2003). *GLYCAM1* gene is expressed during lactation by mammary epithelial cells, as well as in the epithelial cells of the peripheral and mesenteric lymph nodes (Dowbenko *et al*. 1993). The *CD74* gene encodes a glycoprotein with diverse immunological functions in inflammatory diseases. This protein is involved in the formation of chemical complexes of the main class II histocompatibility complex (MHC) and regulates antigen presentation for immune response (Beswick and Reyes, 2009).

The *FCER1G* gene, which encodes the FC receptor for the antibody, is found on the surface of a broad range of hematopoietic cells and binds to the (Fc region) of antibody molecules. By specifically binding the antibody (the Fab region) to a specific antigen, the Fc receptor enables immune cells to respond to the antigens. This mechanism is used by a wide range of cells, including mast cells, basophils, eosinophils, macrophages, neutrophils, and natural killer cells. This function shows that antibody molecules are able to kill bacteria, viruses, and parasites in the body (Garman *et al*. 2001).

Due to the importance of mastitis in the dairy industry and its prevalence in livestock, which reduces milk production in dairy cows and causes great economic losses to farmers, the early identification and treatment of infected cows before the outbreak are important. In this study, four candidate genes that are somehow involved in the immune system were selected to investigate their association with the disease. The expression level of *Lf*, *CD74*, *GLYCAM1* and *FCER1G* genes in cows with mastitis was compared with healthy cows using real-time polymerase chain reaction (PCR).

MATERIALS AND METHODS

Animals and sample collection

In this experiment, the mammary tissue was collected from 10 healthy and 10 Holstein cows with clinical mastitis from Mazandaran meat slaughterhouse (Sari, Iran). The animals studied in the present experiment had a mean age of 5 to 6 years were in the second or third calving and have been bred in the same condition. The diagnosis of healthy and sick cows was made by clinical examinations by a veterinarian and checking the symptoms such as udder/quarter swelling, abnormal milk quality and quantity, and anorexia. The coliform bacteria (*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp.), environmental streptococci (*Strep. dysgalactiae*, *Strep. uberis*) are the major environmental pathogens that cause mastitis in cows. Mammary tissue was collected from sections examined clinically by a veterinarian immediately after slaughter, and tissue fragments without connective tissue and adipose tissue were immediately immersed in frozen liquid nitrogen. In less than an hour, it was transferred to a laboratory and frozen at -80 ˚C.

RNA extraction and cDNA synthesis

30 mg of breast tissue was separated by a sterilized razor and tweezers and placed in a mortar, and after adding liquid nitrogen and freezing the tissue, it was pounded by the handle of the mortar. The total RNA of the tissue samples was extracted using the RNA extraction kit (Yekta Tajhiz Azma, Iran) following manufacturer Instructions. The quantity of the RNA samples was evaluated by spectrophotome Nanodrop (UV-1800/SHIMADZU model, Japan). RNA quality was then evaluated by agarose gel electrophoresis. The first cDNA strand was synthesized using the cDNA synthesis kit (YektaTajhizAzma, Iran). For cDNA synthesis (total vol. 20 µL), 5 µL of RNA were mixed with 10 µL of buffer (2x), 2 µL of reverse transcriptase enzyme, and 3 µL of Rnase-free dH_2O. The average cDNA concentrations were determined by spectrophotometry, after which the cDNA was stored at -20 ˚C until use in the real-time PCR.

Design of primers

Sequences of *Lf*, *CD74*, *GLYCAM1*, and *FCER1G* genes as well as the18S rRNA gene as a reference were extracted from the NCBI database for amplification. The primers were designed using the primer 3 online software. Also, the primers were checked with gene runner software for validation and confirmed with PCR. The binding of primers to the gene sequence, the melting temperature, GC percentage, and the formation of dimer primer and loops and ΔG were checked using Gene Runner software. The sequences of the primers used for real-time PCR are shown in Table 1.

Gene quantification by real-time PCR

At first, gene amplification using PCR was performed to validate the synthesized cDNA using PCR. To prepare the reaction, the Master mix, 2x kit (SinaClon, Iran) was used. According to the kit's recipe, the reaction included: 10 µL of master mix, 100 nanomoles of each forward and reverse primer and 25 nanograms of cDNA, and 7 µL of deionized water mixed. The Real-time PCR reaction was prepared using specific primers and SYBR Green qPCR master Mix (Yekta Tajhiz Azma, Iran) and performed on a Real-time PCR device (Corbett Rotor gene 3000 German). The cycle program consisted: a hot start at 95 ˚C for 5 min, step1, at 95 ˚C for 15 sec, step 2,at 60 ˚C for30 sec, step 3,at 72 ˚C for 45 sec, and the program was repeated for 40 cycles and final extension at 72 ˚C for 5 min. PCR reaction was prepared in 15 µL final volume containing 7.5 µL of SYBR Green qPCR master Mix, 1 µL of cDNA, 1 µL of each primers, and 4.5 µL of RNase-free water in 0.2 µL tubes on a cold rack. How to calculate the fold change is as follows; at first, estimation of delta Ct by subtracting the Ct of the interested gene from the internal standard gene for all samples. $2^{-\Delta Ct}$ was calculated for all patients and healthy samples. Then, the fold changes were measured by dividing the values of $2^{-\Delta Ct}$ of all patient samples by the average of the $2^{-\Delta ct}$ of healthy samples. In this method, the fold change in the healthy group is equal to the one, and in the patient group, it was calculated compared to the healthy group.

Statistical analysis

The relative expression of the genes was evaluated using real-time PCR and the expression level was calculated by a $2^{-\Delta\Delta Ct}$method (Livak and Schmittgen, 2001). Statistical differences between gene expression levels in two groups were examined using Student's t-test using SAS statistical software version 9.1 (SAS, 2003).

RESULTS AND DISCUSSION

The bands of amplified genes after Real-time PCR were observed on the agarose gel (Figure 1).

The amplified bands were in accordance with specific primers for each gene and the desired length, which indicated the function of the primer and the correct performance of the Real-time reaction. The result of *Lf*, *GLYCAM1*, *CD74*, and *FCER1G* genes expression have shown in Figures 2 to 5.

According to the calculation of relative gene expression level by the $2^{-\Delta\Delta Ct}$ method, the expression of *Lf*, *CD74*, and *FCER1G* genes were significantly higher in the patient group than the healthy group.

In contrast, the expression of the *GLYCAM1* gene was significantly lower in the patient group than the healthy group. The expression levels of *Lf*, *CD74*, and *FCER1G* genes in the group of cows with clinical mastitis were 2/5 ($P<0.0003$), 6 ($P<0.0004$), and 2 ($P<0.002$) times higher than healthy cows, respectively.

In contrast, the expression level of the *GLYCAM1* gene was significantly lower (4 times) in the group of cows with clinical mastitis comparing healthy cows ($P<0.0074$). Based on the results of previous studies and considering the role of the mentioned genes, a flowchart was drawn that shows the relationship of these genes in connection with the immune system (Figure 6).

Mastitis is usually the most costly disease causes huge economic losses in the dairy industry. Mastitis is the most common production disease in dairy herds around the world, which can affect milk production.

Table 1 Specifications of designed primers

Primers[1]	Size (bp)	Primer sequence	Temperature (˚C)
Lf (F)	20	5- CGTGGCAGTTGTCAAGAAAG -3	60
Lf (R)	19	5- CAGCACACAAGGCACAGAG -3	60
GLYCAM1 (F)	20	5- CCGATCCCTGACCTCAAATA -3	60
GLYCAM1 (R)	19	5- CCTCCAGGTGGGTTTCATC -3	60
CD74 (F)	19	5- GAGGTCAGAAGCATGGAAG -3	58
CD74 (R)	19	5- ACAGGAAGTAGGCAGTGGT -3	58
FCER1G (F)	19	5- TGGTCCTGCTCTTACTCCTT -3	60
FCER1G (R)	19	5- TGAGTCGGCAGTAGAGCAG-3	60

F: forward and R: reverse.

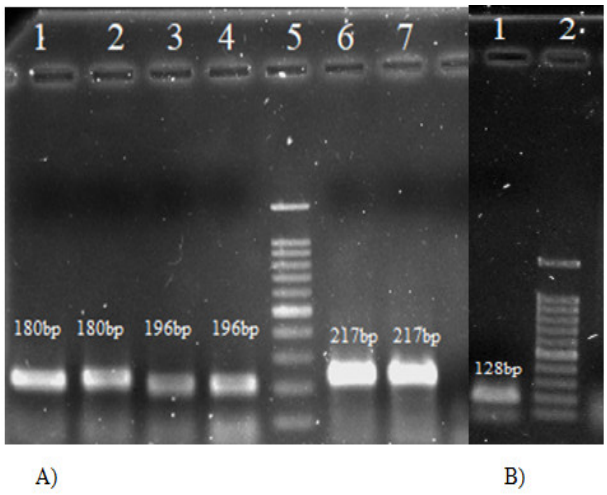

Figure 1 Agarose gel electrophoresis of amplified genes (a and b). a) Line 1 and 2, GLYCAM1 (180 bp); line 3 and 4, CD74 (196 bp); line 5, 100 bp DNA ladder (Sinaclon, Iran); line 6 and 7, Lf (217 bp). b) Line 1, FCER1G (128 bp); line 2, 100 bp DNA ladder (Sinaclon, Iran)

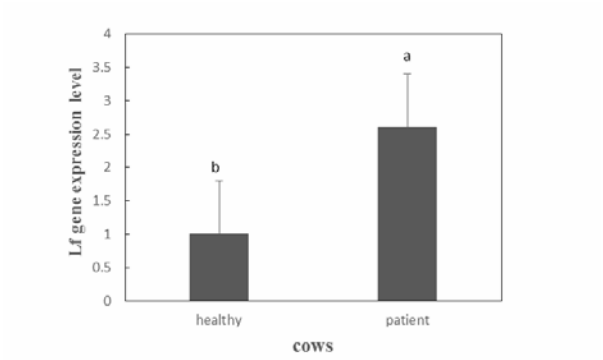

Figure 2 Relative expression level of lactoferrin (*Lf*) gene in healthy and patient cows. Columns represent the fold change of Lf gene between healthy and patient cows. Means with the same letter in each column are not significantly different

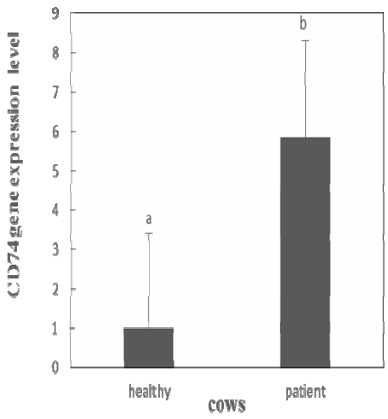

Figure 3 Relative expression level of *CD74* gene in healthy and patient cows. Columns represent the fold change of *CD74* gene between healthy and patient cows. Means with the same letter in each column are not significantly different

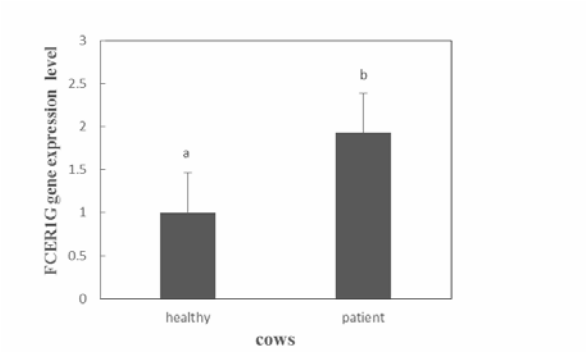

Figure 4 Relative expression level of *FCER1G* gene in healthy and patient cows. Columns represent the fold change of *FCER1G* gene between healthy and patient cows. Means with the same letter in each column are not significantly different

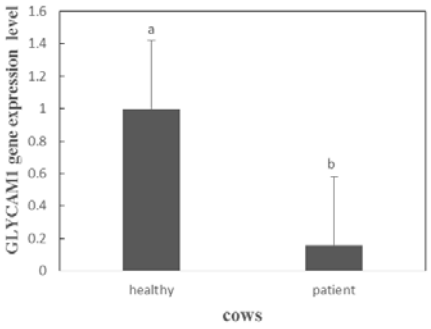

Figure 5 Relative expression level of *GLYCAM1* gene in healthy and patient cows. Columns represent the fold change of *GLYCAM1* gene between healthy and patient cows. Means with the same letter in each column are not significantly different

Decreased milk production per cow due to the clinical and subclinical spread of mastitis, causing economic damage that is caused by the disease. The disease could effect the annual mortality rate and other production (Seegers *et al*. 2003).

In mastitis cows, the immune system is focusing on fighting the infection in the mammary gland and milk production is not enough in them. Resistance to mastitis is one of the economic traits that are controlled in dairy cows by genes related to innate immunity. Study and information on some genes can be important for trying to enhance resistance to mastitis, which is caused by a diverse set of pathogens.

In this study, the relative expression of lactoferrin, *GLYCAM1*, *CD74*, and *FCER1G* genes were compared between cows with clinical mastitis and healthy cows. The results showed that the expression of lactoferrin, *CD74*, and *FCER1G* genes was significantly increased, but the expression of GLYCAM1 was significantly decreased in patient cows compared to healthy ones.

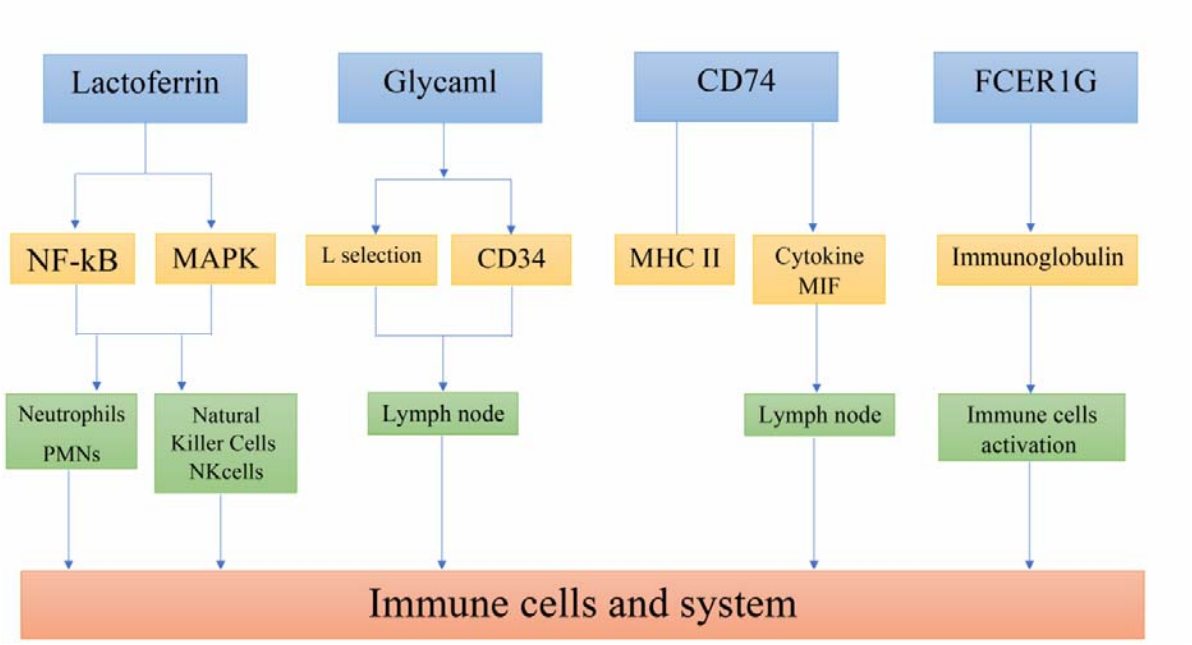

Figure 6 Diagram of the relationship between the genes and the immune system. The lactoferrin gene has a binding site for the NF-κB transcription factor on its promoter, which binds to the NF-κB by increasing inflammation and expression of the lactoferrin gene, resulting NF-κB plays a key role in regulating the immune response to infection. Also, Lf can increse MAPK activity and subsequently, induce an immune response. GLYCAM1 protein in lymphatic organs binds to L-selectin as a molecule that transports lymphocytes from the blood to the secondary lymph nodes
The type II transmembrane protein CD74 is expressed on antigen-presenting cells and was initially demonstrated to function as an MHC class II chaperone. Cell surface CD74 serves as a receptor for the cytokine, macrophage migration inhibitory factor (MIF) on many cell types. In immune cells, MIF binding to CD74 induces a signaling cascade that results in B cell proliferation and regulation of cell survival. The *FCER1G* gene, which encodes the FC fragment receptor for the IgE antibody, which the Fc fragment of antibody bound to its receptor on the surface of phagocytic cells and other immune cells, resulting enables immunity system to respond to antigens

These genes are among the genes associated with the immune system that affect the health and resistance to mastitis in dairy cows and play an important role in regulating the innate and acquired immune response against the entry and trapping of pathogens into the animal body.

For this special role and based on the previously mentioned studies (Hou *et al*. 2000; Garman *et al*. 2001; Beswick and Reyes, 2009; O'Halloran *et al*. 2009; Huiting *et al*. 2017) in this study, a flowchart of the connection of these genes with the immune system was designed to show the role and importance of these genes.

The first defense system against bacterial infection in the mammary gland is innate immunity, which includes antimicrobial peptides such as β-defensin, lactoperoxidase (LPO), and lactoferrin (Kawai *et al*. 2015). Bovine lactoferrin concentrations increase during breast development as well as infection. Lf is a biologically active compound with antibacterial and antioxidant activities on inflammatory reactions.

Lactoferrin is an essential element in innate immunity, which acts as a host non-specific defense against antigens. It has variable concentrations are reported among healthy, subclinical, and clinical mastitis, indicating that there is some association between change in the level of expression of the *Lf* gene and mastitis (Chopra *et al*. 2015).

Bruckmaier (2005) showed that the expression level of bovine Lf mRNA was relatively low in the lactating gland but increased markedly during mammary gland infection. It has been reported that clinical mastitis causes a significant increase in Lf gene expression in dairy cattle (Chaneton *et al*. 2008; Swanson *et al*. 2009). The researchers earlier showed that genetic variations present in the bovine Lf gene can be associated with mastitis (Sharma *et al*. 2013; Chopra *et al*. 2014). Chopra *et al*. (2015) identified the SNP in the promoter region of Lf and reported it may affect the expression of lactoferrin protein, which may lead to different levels of antibacterial and anti-inflammatory activity of the Lf gene. Fonseca *et al*. (2011) showed that lactoferrin gene expression levels were 2.5 times higher in Holstein cow patients with mastitis than in healthy cows. Legrand and Mazurier, (2010) reported that lactoferrin was expressed in the host body one hour after *Escherichia coli* infection and 24 hours after the presence of *aureus* infection (Legrand and Mazurier, 2010). Lactoferrin is an activator of innate immune cells (neutrophils, macrophages, basophils, and mast cells) and an activator of dendritic cells (APCs) in specific defense in epithelial and endothelial cells in mammals.

Therefore, lactoferrin is a molecule that plays a key role against microbes and an essential role in the host's response as a defense barrier against pathogens (Legrand and Mazurier, 2010).

In previous studies, it was found that lactoferrin gene expression, as well as Lf protein concentration in milk, was significantly increased in dairy cows with mastitis. Therefore, bovine lactoferrin has been considered an important factor in the innate defense mechanisms of the mammary gland against mastitis (Huang *et al*. 2011; Pawlik *et al*. 2014). The immunomodulatory property of lactoferrin is related to the molecular patterns associated with pathogens by binding to the lipopolysaccharide moiety of the organism. This binding ability converts lactoferrin into an anti-inflammatory molecule that can protect the host from harmful immune responses (Legrand and Mazurier, 2010).

Asselstine *et al*. (2019) studied functional candidate genes of *GLYCAM1*, *CD74*, and *FCER1G* based on the criteria of being highly differentially expressed (DE) between healthy and mastitic groups. These genes are involved in metabolic pathways that are relevant to the inflammatory process.GLYCAM1 is down regulated and under-expressed in a mastitic group compared to the healthy.

The *GLYCAM1* gene is a family of glycoproteins that are part of the membrane of milk fat cells and this gene is expressed specifically in the mammary gland during breast feeding and in peripheral and mesenchymal lymph node endothelial cells. This glycoprotein acts as a ligand to bind to L-selectin and is an intermediate in the transport of blood lymphocytes to the secondary lymph node (Groenen *et al*. 1995; Hou *et al*. 2000). GLYCAM1 can not mediate the trafficking of blood-borne lymphocytes into the milk (Le Provost *et al*. 2003). LeProvost *et al*. (2003) used a macro array method to study the expression of various genes during lactation and pregnancy in the mammary gland of the breast and found that the mRNA level of the *GLYCAM1* gene was higher during lactation than during pregnancy and also the expression of this gene in the uterus, lung, lumen and the endothelial surface of the peripheral mesenteric lymph nodes have been reported (Le Provost *et al*. 2003). *GLYCAM1* gene was located in quantitative trait locus (QTL) regions previously known to be associated with mastitis, specifically clinical mastitis. Since mucins play an important role in the protection and inflammatory responses, for this reason a QTL region within the *GLYCAM1* gene could be responsible for the phenotypic variation observed in healthy and mastitis cows (Asselstine *et al*. 2019). Asselstine *et al*. (2019) studied the expression of the *CD74* gene in the breast tissue of two groups of healthy and mastitis cows by RNAseq method. The results showed that the expression of this gene in the patient group was almost five times that of the healthy group. CD74 upregulated and over expressed in a mastitic group compared to a healthy. The CD74 gene encodes a glycoprotein with diverse immunological functions and plays a role in antigen presentation pathways (Beswick and Reyes, 2009; Na *et al*. 2017). This gene is directly related to the major histocompatibility complex (MHC) molecules, which are an essential part of the immune system that facilitates antigen recognition, presentation, and activation of an acquired immune response (Behl *et al*. 2012). The most well-known function of CD74 is to regulate the transfer of biocompatible proteins class II (MHCII) in antigen-presenting cells (APC). MHCII processing and regulation cannot properly occur in the absence of CD74 (Beswick and Reyes, 2009). CD74 is involved with antigen-presenting cells (APC), which aid in the protection of the host as they signal $CD4^+$ T helper cells, which kill the pathogen as part of the immune response (Su *et al*. 2017). Some studies confirmed that CD74 is involved with inflammatory diseases (Beswick and Reyes, 2009; Na *et al*. 2017; Su *et al*. 2017). The presence of variants in the sequence of this gene may lead to cows that are susceptible to mastitis infections (Asselstine *et al*. 2019).

The *FCER1G* gene, which encodes the FC receptor for the antibody, specifically binds the antibody to a specific antigen, the FC receptor enables immune cells (mast cells, basophils, eosinophils, macrophages, neutrophils, and lethal T cells) to respond to the antigens (Garman *et al*. 2001; Woof and Burton, 2004). Asselstine *et al*. (2019) studied the expression of the *FCER1G* gene in the breast tissue of two groups of healthy and mastitis cows by RNAseq method. It showed that the expression of the desired gene in the patient group was significantly higher than in the healthy group. The immunoglobulin molecules are able to eliminate bacteria, viruses, and parasites from the body and for the *FCER1G* gene, the immunoglobulin of interest is IgE, which helps to protect against parasitic infections (Woof and Burton, 2004). Asselstine *et al*. (2019) combined the results from transcriptomic and functional analysis and identified that *CD74*, *GLYCAM1*, and *FCER1G* genes are associated with mastitis susceptibility and resistance.

CONCLUSION

Despite several studies on various genes related to resistance to mastitis, the present study is the first report on the expression of *Lf*, *GLYCAM1*, *CD74*, and *FCER1G* genes on the mammary epithelial tissue of Holstein cow in Iran. Due to the importance of mastitis in the dairy industry and its prevalence in livestock, which reduces milk production in dairy and causes great economic losses to farmers, early

identification and treatment of infected cows before the outbreak is important. Since these four selected genes are associated with immune responses associated with inflammatory diseases, including mastitis, they can be used as diagnostic markers for early detection of the disease after confirming the results of this study in larger populations. Considering the important role of these genes in immune responses, it is recommended to identify single nucleotide polymorphisms or deletions/insertions in the coding regions of these genes and investigate their relationship with mastitis.

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