Association assessment of single nucleotide polymorphism in Forebrain Embryonic Zinc Finger-Like (*FEZL*) gene with mastitis susceptibility in Holstein cattle (*Bos taurus*)

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SUMMARY

The accentuation on improving the dairy industry has been to concentrate on breeds that have high milk yields, mainly Holstein. Bovine mastitis continues to be the most powerful disease threatening dairy industry worldwide. It remains the major challenge to the overall dairy industry regardless of the far reaching execution of mastitis control strategies. The immune response to mastitis is complex and requires the development of a sophisticated regulatory system to carry out functions at signal-specific and gene-specific levels which, can be used in dairy breeding selection. The aim of this study is to find out whether a three base insertion located at the glycine stretch of forebrain embryonic zinc finger-like (*FEZL*) gene, a transcription factor which possibly has an immune function, influences the milk somatic cell count (SCC) and mastitis susceptibility in Egyptian dairy cattle. Furthermore, relationships were explored between *FEZL* gene and its level of expression and semaphorine 5A (*SEMA5A*) gene level of expression, an immune gene through which FEZL has its immune function. Both FEZL and SEMA5A have an important antimicrobial role by controlling the neutrophilic migration to the site of mammary gland infection. Blood was collected from the jugular or mammary gland vein of 112 normal and mastitic unrelated Egyptian Holstein-Friesian dairy cattle. Single-strand conformation polymorphism (SSCP) analysis was carried out to detect single nucleotide polymorphism (SNP) in the amplified glycine stretch of FEZL. The expression levels for *FEZL* and *SEMA5A* were assessed in milk samples. Our sequencing results proved that cattle carrying *FEZL* gene with glycine stretch containing 13 glycine residues have higher SCC and are more susceptible to mastitis than 12G *FEZL* cattle. Moreover, mastitis and high (SCC) were associated with up-regulation in the level of expression of both *FEZL* and *SEMA5A* genes. In conclusion, utilizing the SNP of *FEZL* gene before genetic selection for the dairy industry may be useful to control the incidence of mastitis.

KEY WORDS

Bovine mastitis, *FEZL*, *SEMA5A*, Polymorphism, SSCP.

INTRODUCTION

In numerous nations, the accentuation on enhancing dairy generation has been to concentrate on breeds that have high milk yields, mainly Holstein[1]. Mastitis is one of the most common diseases influencing up to 40 percent of cows within a herd[2]. It remains the major challenge to the overall dairy industry regardless of the far-reaching execution of mastitis control strategies[3]. The urgency for identifying hereditary markers associated with mastitis resistance has taken two ways: scanning for quantitative trait loci (QTL) as well as SNP in candidate genes[4]. SNPs are the most abundant polymorphism in eukaryotic genomes that can be used as a superlative marker type for characterization of economically important traits[5].

The immune response to mastitis is complex and requires the development of a sophisticated regulatory system to carry out functions at signal-specific and gene-specific levels. It includes various different genes and cellular pathways, so genes coding for immune factors that detect and eliminate pathogens are potential markers[6]. Forebrain embryonic zinc finger-like gene (*FEZL*) was identified as a QTL influencing mastitis resistance[7]. It belongs to the krueppel C2H2-type zinc-finger protein family and contains six C2H2 type zinc-finger domains and a glycine stretch[8]. It acts as a transcription factor, repressor, which binds to 5'-GCAG-3' core sequence in target genes[9]. Semaphorine 5A (*SEMA5A*) is one of the target genes of FEZL. Semaphorins are an extensive family of widely expressed secreted and membrane-associated proteins[10].

Both FEZL and SEMA5A have an important antimicrobial role by controlling the neutrophilic migration to the site of mammary gland (MG) infection. When cows are infected with mastitis, FEZL, as a transcription factor, is able to induce tumor necrotic factor-α (TNF-α) and interleukin-8 (IL-8) through enhancing SEMA5A[9]. IL-8 is responsible for controlling the number of neutrophils migrating to the site of infection which is critical for the resolution of mastitis[11].

The length of the glycine stretch in *FEZL* may influence its transcription activity. 12G *FEZL* promotes greater *TNF-α* and *IL-8* expression, through the promotion of higher *SEMA5A* expression than 13G *FEZL*. This demonstrates that 12G *FEZL* may impacts to clinical mastitis more than 13G

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FEZL where the impaired TNF-α and IL-8 expression controlled by *FEZL* and *SEMA5A* can clarify susceptibility of 13G *FEZL* cattle to mastitis[9].

MATERIALS AND METHODS

Blood samples

Blood was collected from the jugular or mammary gland vein puncture in vacutainer tubes containing EDTA as anticoagulant from 112 normal and mastitic unrelated Egyptian Holstein-Friesian dairy cattle (*Bos taurus*) randomly selected from different farms in different governorates (Giza, Fayoum, Beheira and Alexandria) in Egypt to maximize genetic diversity. Samples were kept at -20°C till used.

Genomic DNA isolation

Genomic DNA was isolated from blood using Blood DNA preparation kit (Jena bioscience, Cat. No. PP-205s, Germany) and the DNA concentration was adjusted to 20 ng/μL[12].

PCR amplification

Genomic DNA spanning the glycine stretch of *FEZL* gene was amplified by PCR to amplify a 229 bp region that contains the SNP as determined in Holstein-Friesian dairy cattle population using High yield Master Mix (Jena Bioscience, Germany) and the primer sequence for the *FEZL* gene were used according to Sugimoto and Sugimoto; [forward: 5'-TCCAAGACGCTGCTCAGTTA-3'; reverse: 5'CCACAGCCTGGTTGATGAC-3'][9,13]. Conditions for amplification were as follows: an initial denaturation occurred at 95°C for 5 minutes, followed by 35 cycles of 95°C denaturation for 1 minute, 62°C annealing for 1 minute and 72°C extension for 1 minute and a 10 minutes final extension step at 72°C. The specificity and yield of PCR products were checked by electrophoresis on a 2% agarose (Bioshop, Canada) in 1X TAE buffer (Tris base, glacial acetic acid, EDTA, DDW PH 8.3, Bioshop, Canada), stained with ethidium bromide (Sigma Aldrich, USA).

Single-strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) analysis was carried out to detect SNP in the amplified glycine stretch of *FEZL*. Ten microliters of the PCR product was mixed with 10 μl of the denaturing loading buffer (formamide, bromophenol blue and xylene cyanol, Bioshop, Canada), denatured for 10 min at 98°C, rapidly chilled on an ice block and kept in -20°C deep freezer for 15 minutes then loaded onto 15% polyacrylamide gel (39:1) (6 ml polyacrylamide 50%, 2 ml 10X TBE, 12 ml D.W, 45 μl APS, 25 μl TEMED, Bioshop, Canada). Electrophoresis was performed in 1X TBE buffer (Tris base, Boric acid, EDTA, DDW PH 8.3, Bioshop, Canada) in the following conditions: 80V at 4°C for 10 hrs. SSCP gel was detected using silver nitrate (Vivantis, Malaysia) by rapid silver staining protocol and the patterns of DNA were observed as described by Blum *et al.*[14].

Sequencing of the selected genotypes

Genotyping was performed by sequencing reactions of purified DNA using Big Dye TM Terminator v3.1 Cycle Sequencing Kits on an ABI PRISM 3730XL Analyzer (Applied Biosystems, USA)[15]. Sequences of *FEZL* amplified fragments were aligned using Nucleotide BLAST program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST) for sequence homology searches against GenBank databases, *Bos tauras FEZL* gene (NP 001033287.1) and *mutant Bos taurus FEZL* gene (AAV85458.1).

Milk samples

Milk samples were collected aseptically and kept at 4°C where they were processed within 24 h to measure the SCC by Bentley Soma Count 150 (Bentley, USA) as described by Zecconi *et al.*[16]. Animals were divided according to their SCC into two main groups[17]:

(1) **Normal group:** Cows for which the $\log^{10}$ (SCC) values were below 200 X 10^3 cells/ml were classified to the Low-SCC class (Normal) (**72 Animals**).

(2) **Mastitic group:** Cows for which the $\log^{10}$ (SCC) values were equal to or higher than 200 X 10^3 cells/ml were classified to the High-SCC class (Mastitic) either subclinical or clinical mastitis (**40 Animals**).

Isolation of total RNA and reverse transcription

Total RNA was extracted from milk using QIAamp RNA Blood Mini Kit (QIAGEN, Cat. No. 52304, Germany) after sedimentation of milk leukocytes. To remove any contamination by genomic DNA, the samples were submitted to digestion with DNase (RNase-free DNase Set, Qiagen). The purity and concentration of the isolated RNA were ascertained spectrophotometrically using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). RT-PCR was performed using RevertAid Reverse Transcriptase, 200 U/μl (Thermo Scientific, Cat. No. EP044, USA) following the guidelines provided[18].

Quantitative real-time RT-PCR (qPCR) of FEZL and SEMA5A genes

Based on the genomic sequences of *FEZL* and *SEMA5A* mRNA available in GenBank; (NM_001038198.2) and (XM_002696441.2) respectively, the forward and reverse primers were designed by Primer 3-program (http://frodo.wi.mit.edu/cgibin/primer3/primer3_WWW.cgi). For *FEZL* [forward: 5'- AACGTTTCCTCGATGACTGG-3'; reverse: 5'-GTGACAGGCTGGGGTTAAAA-3'] and for *SEMA5A* [forward: 5'-CAAGTGGCTCAACGAACCAA-3'; reverse: 5'-AG TAGAAAGGCACCTCACCC-3']. The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference gene and amplified by the following primers according to Sugimoto *et al.* [forward: 5-' GCCCTCAACGACCACTT TGT -3'; reverse: 5'- CCTGTTGCTGTAGCCAAATTCA- 3'][9]. Real-time PCR was performed by Luminaris Color HiGreen Low ROX qPCR Master kit (Thermo Fischer Scientific, Cat. No. K0371, USA). The cDNA was amplified by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 45 s[19]. The fluorescence intensity was acquired at the annealing step of each amplification cycle and the specificity of the amplicons was checked by performing the "melting curve" analysis of all samples through one cycle of 95°C for 15 s, 55°C for 15 s and 95°C for 15 s. Each qRT-PCR was performed in triplicates with no template control (NTC) included in each experiment. The fold change over control samples was calculated using ΔCT, ΔΔCT and $2^{-\Delta\Delta CT}$ by Mxpro software Stratagene[20].

Statistical analysis

The statistical analysis for all studied genetic locus was carried out according to Zwierzchowski *et al.*[21]. Observed allelic and genotypic frequencies were calculated using Hardy-Weinberg equilibrium where it was assessed by Chi-square test for determination of the independence between allelic and genotypic frequencies. SCC data were analyzed by Least Square Means with associated standard error (LSM ± S.E) using the General Linear Mode (GLM) procedure of the Statistical Analysis System (SAS/STAT program, version 7, 2002). Differences between the groups were assessed using student (t) test. Statistical significance was declared at a P level of < 0.05 by Duncan's multiple comparisons.

RESULTS

PCR amplification and polymorphism in *FEZL* gene

Glycine stretch of *FEZL* gene was successfully amplified giving rise to 229 bp amplicon (Figure 1A). The SSCP results revealed the presence of three clearly definable SSCP patterns (A, B and C) (Figure 1B).

Sequencing of *FEZL* gene

The sequencing results of the specific purified PCR product of *FEZL* gene for both normal and mastitic samples revealed the presence of two isoforms of *FEZL* gene according to the length of its glycine stretch (Figure 2).

Genotypes and alleles frequency for *FEZL* gene

The allelic and genotypic frequencies of *FEZL* SNP in normal and mastitic animals are shown in (Table 1) and (Table 2), respectively. Allele A showed the highest frequency (0.923 and 0.892 in normal and mastitic animals, respectively). The obtained exact P values for X^2 test in all populations confirmed the accordance with the Hardy-Weinberg distribution in all investigated animals.

Impact of *FEZL* genetic polymorphism on SCC

Results of association analysis of *FEZL* SNP with SCC revealed that the CC genotype has the highest mean value of SCC, while, the AB genotype has the lowest mean value of SCC as shown in (Table 3).

Expression of *FEZL* and *SEMA5A* genes

Quantitative RT-PCR analysis showed the presence of significant elevation in the expression level of both *FEZL* and *SEMA5A* genes in mastitic samples compared to normal samples by three and two folds, respectively (Figure 3).

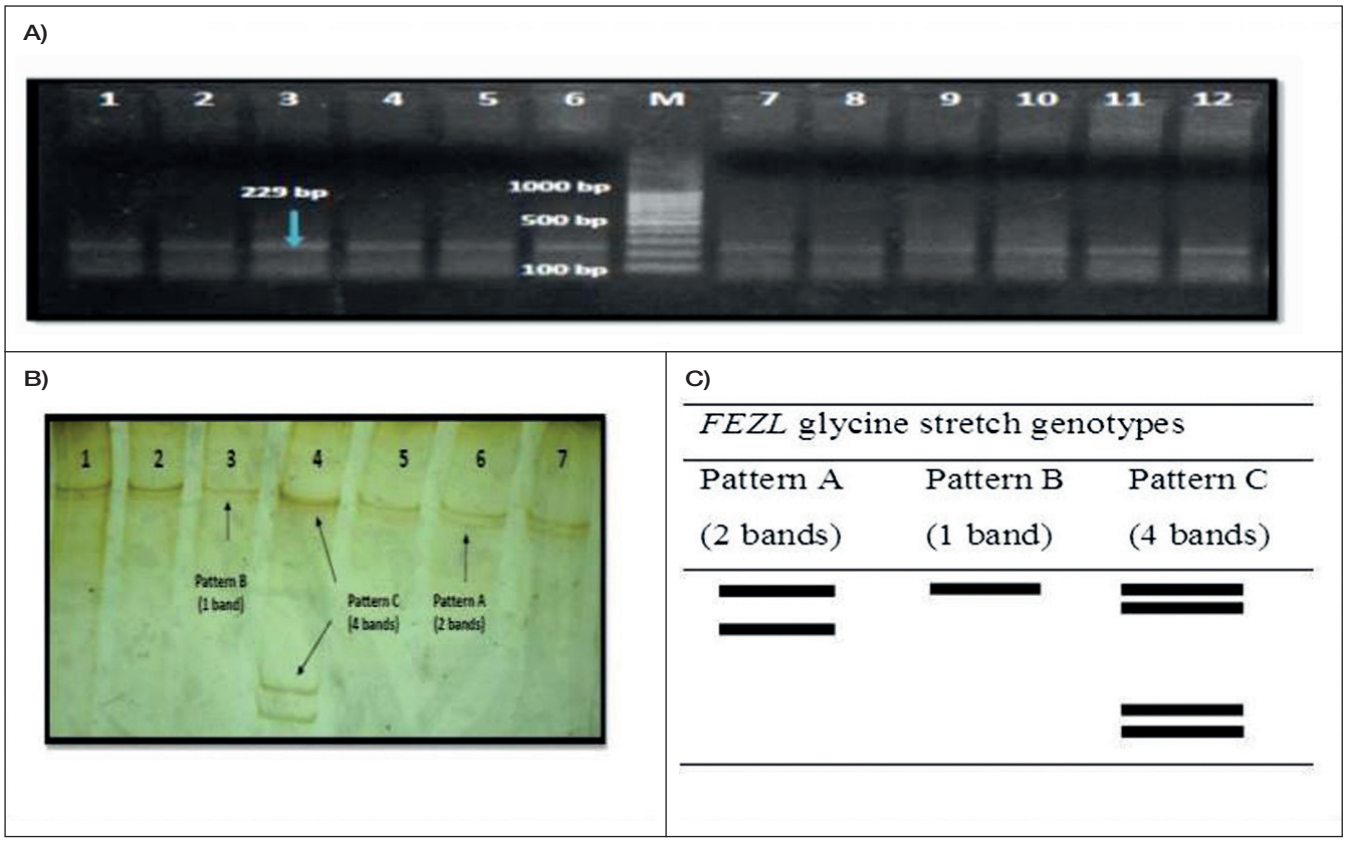

Figure 1 - PCR-SSCP for *FEZL* gene.
A) Agarose gel (2%) showing PCR products of Forebrain Embryonic Zinc Finger-Like gene (*FEZL*) of normal and mastitic samples. Lane M: 100bp DNA molecular weight marker; Lanes (1-6): Normal samples; Lanes (7-12): Mastitic samples. Single specific bands of 229 bp were clear in all lanes.
B) PCR-SSCP for *FEZL* gene in normal and mastitic samples. 15% non-denaturing Polyacrylamide gel for FEZL gene: Denatured PCR products of *FEZL* gene separated in 15% non-denaturing PAGE (39:1) stained with silver. Lanes (1-4): Normal samples; Lanes (5-7): Mastitic samples. Lane 1, 2, 5, 6 and 7: Pattern A (2 bands); Lane 3: Pattern B (1 band); Lane 4: pattern C (4 bands).
C) Schematic representation of SSCP patterns for indicated *FEZL* gene.

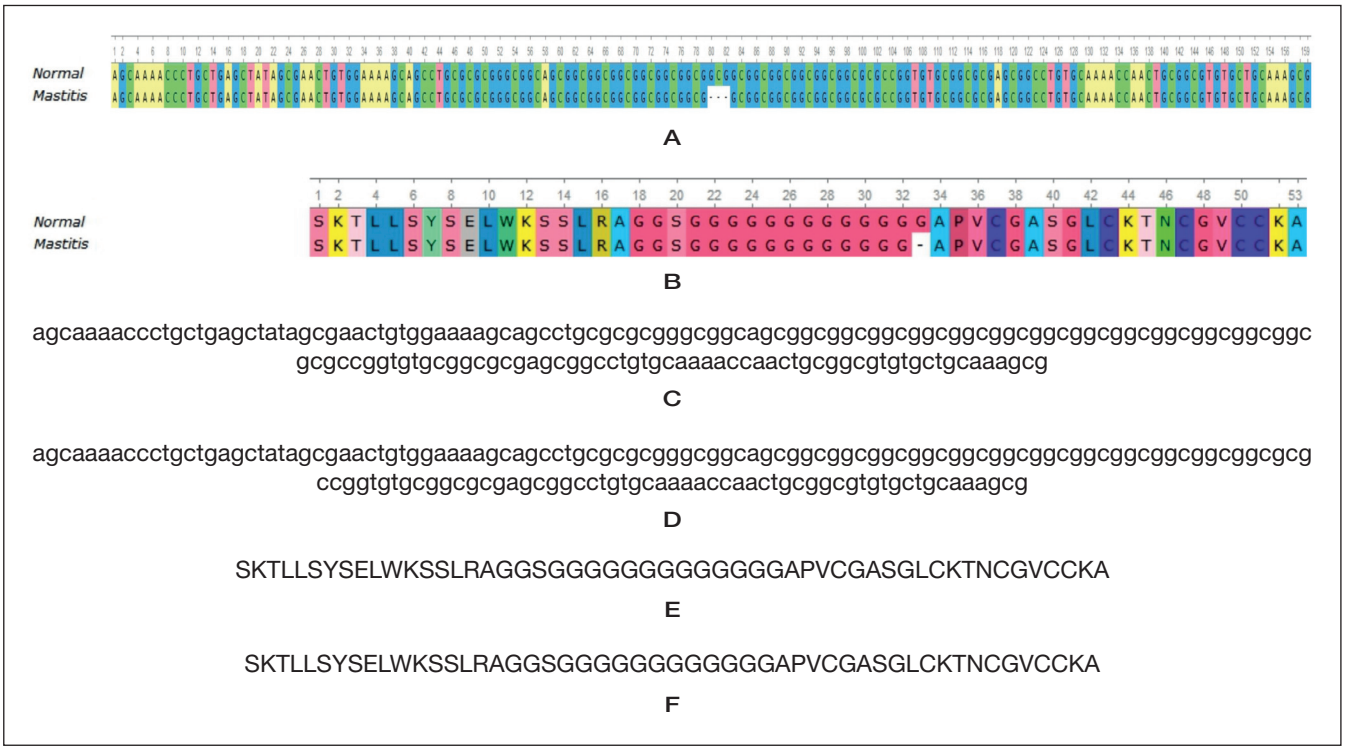

Figure 2 - The sequencing results of the *FEZL* amplicons in both normal and mastitic samples.
(A) The Clustal W alignment of the *FEZL* sequence obtained by sequencing of the target gene fragment in both normal and mastitic samples; **(B)** The Clustal W alignment of the deduced amino sequences in both the normal and mastitic samples; **(C)** Sequencing results of the normal cattle; **(D)** Sequencing results of the mastitic cattle; **(E)** The deduced amino acid sequence of the *FEZL* fragment in the normal samples; **(F)** The deduced amino acid sequence of the *FEZL in mastitic samples*.

Table 1 - Allelic and genotypic frequencies of *FEZL* gene and Hardy-Weinberg Equilibrium (HWE) in normal samples.

Glycine stretch in *FEZL* gene in normal samples (N=72)									
	Genotypes						Allelic frequency		
	AA	AB	AC	BB	BC	CC	A	B	C
Observed number	66	5	0	0	0	1			
Expected number	61.33	4.52	1.86	0.083	0.068	0.014	0.923	0.034	0.014
Genotype frequency	0.917	0.069	0	0	0	0.014			
$X^2 = 71.85$									

X^2 = Chi square test.
A degree of freedom (df) of 3 [number of genotypes (6) - number of alleles (3)], Chi-square value shows a difference ($P < 0.05$) when compared with the tabulated Chi-square value (7.81), so the deviation between observed genotypic frequencies and those expected under HWE in normal samples was statistically significant ($P < 0.05$) suggesting that Holstein-Friesian population sample was not in equilibrium for glycine stretch of *FEZL* locus.

Table 2 - Allelic and genotypic frequencies of *FEZL* gene and Hardy-Weinberg Equilibrium (HWE) in mastitic samples.

Glycine stretch in *FEZL* gene in mastitic samples (N=40)									
	Genotypes						Allelic frequency		
	AA	AB	AC	BB	BC	CC	A	B	C
Observed number	34	1	0	0	0	5			
Expected number	29.75	0.86	8.63	0.006	0.125	0.625	0.8625	0.0125	0.125
Genotype frequency	0.85	0.025	0	0	0	0.125			
$X^2 = 40.01$									

X^2 = Chi square test.
A degree of freedom (df) of 3 [number of genotypes (6) - number of alleles (3)], Chi-square value shows a difference ($P < 0.05$) when compared with the tabulated Chi-square value (7.81), so the deviation between observed genotypic frequencies and those expected under HWE in mastitic samples was statistically significant ($P < 0.05$) suggesting that Holstein-Friesian population sample was not in equilibrium for glycine stretch of *FEZL* locus.

Table 3 - LSM and SE for SCC across glycine stretch of *FEZL* gene genotypes.

	Genotypes		
	AA	AB	CC
Mean±SE	454.27±43.7[b]	226.16±4.9[c]	674.92±48.1[a]

[a-c] Means with different superscripts differ (P < 0.05).The mean values indicate the presence of a difference (P < 0.05) in SCC among the three genotypes of glycine stretch of *FEZL* gene where the CC genotype has the highest mean value of SCC.

DISCUSSION

Exploring the genetic background of economic traits is a major goal in animal genetics and breeding programs. SNPs have been the markers of choice in recent years, due to their high stability, density and their highly automated detection techniques[22]. Several immune genes and pathways, including recruitment and functionality of neutrophils and humoral or cellular adaptive immune response, have proved to play an important role in the severity of mastitis[23]. Therefore, the present study was conducted to illustrate the genotyping of a gene which is responsible for the immune response of animal against mastitis, *FEZL* gene, and to correlate the effect of its genetic variability with SCC. Milk SCC has been identified as the gold standard examination that measures the degree of udder inflammation. SCC has a very strong genetic correlation with mastitis ranging between 50 and 80% with an average of 78%[24].

FEZL gene contains six C2H2 type zinc-finger domains and a glycine stretch where the three base insertion resulted in an extension of 12 glycines (12G) to 13 glycines (13G). This polymorphism was reported to be significantly associated with SCC and susceptibility to mastitis in dairy cattle in Japan[8,9].

Our results showed three clearly definable SSCP patterns (A, B and C). The frequencies of the three genetic variants were 0.917, 0.069 and 0.014 for normal animals and 0.85, 0.025 and 0.125 for mastitic animals, respectively. The difference between observed genotypic frequencies and those expected under HWE were statistically significant (P ≤ 0.05), suggesting that the Egyptian Holstein-Friesian population sample was not in equilibrium for glycine stretch of *FEZL*, therefore there is a selective advantage for the glycine stretch locus of *FEZL* gene and it could be a putative locus for marker-assisted selection. These results came in the same line with Sugimoto *et al.* who correlated the presence of 3 genotypes of *FEZL* to the glycine stretch in Japan, 12G/12G, 12G/13G and 13G/13G *FEZL*[9]. Sugimoto *et al.* on their studies on genotyping of *FEZL* in 918 sires found that 97 sires had a heterozygous 12G/13G genotype and 821 sires had a homozygous 13G/13G genotype[8].

The major allele which appeared in only two of the three genotypes is allele (A) where it has the highest frequency (0.923 and 0.892 in normal and mastitic animals, respectively). On the other hand, allele (B) and (C) appears in lower frequencies (0.034 and 0.0125) and (0.014 and 0.125) in normal and mastitic animals, respectively. Allele (B) shows the least frequency in mastitic animals, while allele (C) shows the least frequency in normal animals. Moreover, the presence of allele (B) is associated with lower SCC while the presence of allele (C) is associated with higher SCC. The above-mentioned results revealed that the presence of (C) allele may be associated with both higher susceptibility to mastitis and higher SCC. These results agreed with Sonstegard *et al.* which stated that 12G allele frequencies were less than 3% in Holstein sires in North America[25]. This allele tended to have lower SCC during the first lactation.

The sequencing results of the specific purified PCR product of *FEZL* gene for both normal and mastitic samples revealed the presence of two isoforms of *FEZL* gene according to the length of its glycine stretch. These results were found to be compatible with that of the SSCP results. Samples which have the pattern C (4 bands) on SSCP gel were proved to contain a three-base insertion in glycine stretch which results in its extension from 12G to 13G. On the other hand, samples which have pattern A (2 bands) and pattern B (1 band) on SSCP gel contain only 12 glycine residues in their glycine stretch.

Considering the relationship between length of glycine stretch and SCC, our results proved that CC genotype, which carries 13G *FEZL*, has the highest mean value of SCC, while AA and AB genotypes, which carry 12G *FEZL*, have lower mean values of SCC suggesting that 13G *FEZL* is more susceptible to mastitis than 12G *FEZL*. These results came in

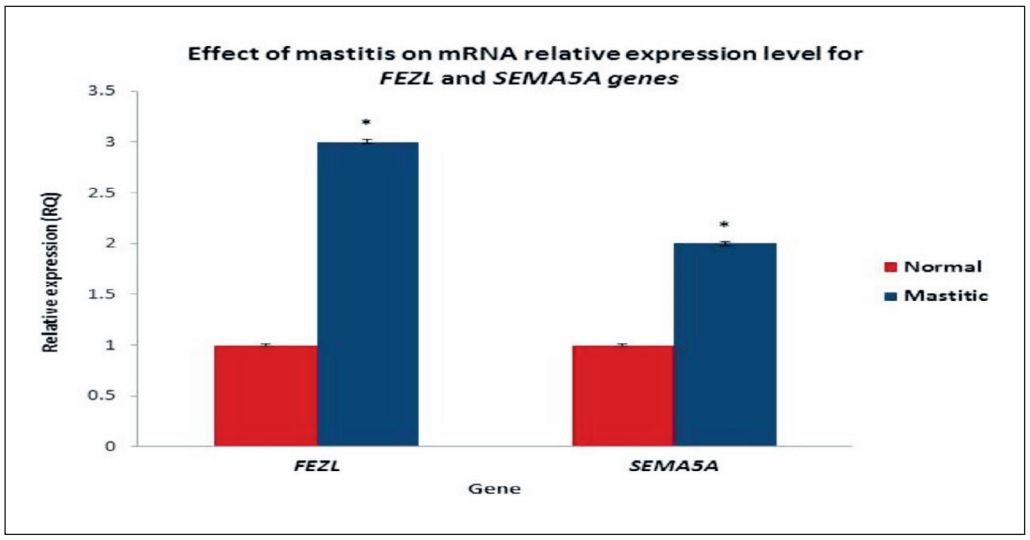

Figure 3
The effect of mastitis on the mRNA expression level of *FEZL* and *SEMA5A* genes. RT-PCR results of milk samples: The mRNA relative expression of *FEZL* and *SEMA5A* genes. Data were expressed as means ± SEM of triplicate experiments. Groups having (*) are significantly different compared with their control groups at p <0.05.

similar line with many authors who reported that there is a significant correlation between different genotypes and SCC and mastitis susceptibility. Sugimoto *et al.* correlate higher mastitis susceptibility of 13G *FEZL* than 12G *FEZL*[9]. Moreover, Sugimoto *et al.* reported that at almost every age, the proportion of affected heterozygous 12G/13G daughters was significantly lower than that of affected homozygous 13G/13G daughters and that 13G/13G *FEZL* cattle have twice the rate of susceptibility to mastitis than 12G/12G *FEZL* cattle[7]. These findings might confirm that *FEZL* affects the animal susceptibility to mastitis as well as SCC.

Gene expression profiling of MG tissue is a powerful tool that has been used to catalog the genes expressed during lactation[24]. Numerous studies have examined gene expression in the bovine MG by performing mammary biopsies; however, such techniques are invasive, disturb the normal lactation process, are labor-intensive and are costly. An alternative sampling procedure has been proposed by isolating mRNA directly from somatic cells that are naturally released into milk during lactation[25].

Our RT-PCR results revealed a significant up-regulation of the expression level of *FEZL* and *SEMA5A* by three and two folds, respectively. In agreement, the induction of expression level of *FEZL* and *SEMA5A* has been reported in mastitic cattle where *FEZL* expression was greater in the mastitic quarter than in the cured quarter of the same animal[10]. Moreover, it has been indicated that 12G *FEZL* stimulates greater *SEMA5A* expression than 13G *FEZL* and that 12G/13G *FEZL* cattle have greater *SEMA5A* expression than in 13G/13G *FEZL* cattle[10]. The enhancement of *FEZL* expression in the MG by intramammary infection is due to its important antimicrobial role where it is responsible for the control of cytokine expression. When cows are infected with mastitis, FEZL induces TNF-α and IL-8 through enhancing SEMA5A, which represents a plausible mechanism to explain the enhanced antimicrobial activity of FEZL-expressing cells[26]. This induced production of IL-8 by TNF-α can regulate the number of neutrophils migrating to the site of infection[27]. Neutrophils are highly effective phagocytes for clearing infecting bacterial pathogens from host tissue[28]. During mastitis, neutrophils become the predominant (>95%) cell type in milk[29]. In contrast, our RT-PCR run against other study which did not observe significant differences in the expression level of *FEZL* and *SEMA5A* between normal and mastitic cattle[30].

It seems that the increase in the level of expression of both *FEZL* and *SEMA5A* genes in mastitic Egyptian dairy Holsteins might improve the significant correlation between *FEZL* and animal immune system. Moreover, the high SCC associated with animals carrying a three-base insertion in glycine stretch of *FEZL* gene could provide a new avenue for MAS programs in Egypt.

CONCLUSIONS

The current study can conclude that cattle carrying *FEZL* gene with glycine stretch containing 13 glycine residues have higher SCC and are more susceptible to mastitis than 12G *FEZL* cattle. The higher susceptibility to mastitis might be due to impaired TNF-α and IL-8 expression controlled by FEZL and SEMA5A. This study may be considered the first record which reported the genetic variation *FEZL* gene in the Egyptian Holstein-Friesian cattle. Thus, in order to control the incidence of mastitis in Egypt, we recommend utilizing the SNP of *FEZL* gene before genetic selection for the dairy industry.

AUTHOR CONTRIBUTIONS STATEMENT

Said M. Zaki conceived and designed the experiment, Ghada E. Ali executed the experiment, Ghada E. Ali and Marwa A. Ibrahim analyzed the results. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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