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DNA MICROARRAY META-ANALYSIS AND MIRNAS TARGET PREDICTION ADDRESS THE COMPLEXITY OF MASTITIS CONTROL IN BOVINE

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ABSTRACT

Bovine mastitis is a widespread disease in dairy cows, and is often caused by bacterial mammary gland infection. Reduced milk production, reduced milk quality, additional labor costs, increased cattle replacement rates, veterinary costs, and treatment costs all strengthen the importance of effective disease control. In this study to uncover the genes involved in the development of mastitis, was performed a meta-analysis using publicly available GEO microarray datasets (GSE15019, GSE24217, GSE24560, GSE25413 and GSE50685). The datasets were processed using the metaDE package in R language was used to do meta-analysis. In this way, Fisher's exact test was used. Next, clustering analysis was performed to detect the distinguishing effect of metaDE on differential expression in different sample groups. The threshold for DEGs was set at a false discovery rate (FDR) of <0.01 to statistically screen out significant difference between gene expressions. The results of DNA microarray meta-analysis shown that a total of 32 genes were identified to be differentially expressed (P <0.01) across GEO datasets. In this study EST-based homology search is applied to find potential miRNA in bovine mastitis. For blast between EST & microRNA, Blastn program version 2.3.1 was used to predict miRNA target. In this study, 940 miRNAs were predicted from 32 genes were identified to be differentially expressed by using a bioinformatics-based gene search. The results of the analysis of miRNAs showed that mRNAs bta-mir-1777b, bta-mir-1777a, bta-mir-2382-3p, bta-mir-2382-5p, bta-mir-671, bta-mir-2888 and bta-mir-370 have the highest frequency. These miRNAs have the most effect on mastitis through the four cell signaling pathways Toll-like receptor signaling pathway, pathway in cancer, Estrogen signaling pathway and Progesterone-mediated oocyte maturation (Diagram 2-5). These miRNAs result in this pathway through cell cycle processes, DNA repair and cell proliferation, glycolysis, gluconeogenesis, and apoptosis. Overall, findings from this study will accelerate the way for further researches of miRNAs and their functions in mastitis.

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Introduction

Mastitis is one of the serious economic problems in dairy cattle and is also a complex and costly disease. Reduced milk production, reduced milk quality, additional labor costs, increased cattle replacement rates, veterinary costs, and treatment costs all strengthen the importance of effective disease control [1]. To control and manage mastitis genetically, many quantitative genetic studies on mastitis have been done, but most of them indicate that heritability of this trait is low [2]. This is why controlling and deciphering this disease using new technology data come to play. MicroRNAs (miRNAs) contain about 19-24 nucleotides that result in expression of the gene expression at transplantation levels, by binding to target mRNAs to initiate mRNA isolation or inhibit mRNA translation [3-6]. Many studies have been shown that miRNAs play an

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important and essential role in the biological processes of plants and animals [7-9]. As a result of various studies, it has been shown that microRNAs are small and long acting through specific sequences with regulating regions, and thus these microRNAs are involved in gene expression regulation [3, 10-12] miRNAs are known in the cells and in liquids such as blood, urine, saliva, amniotic fluid, and milk. In liquids, miRNAs are enclosed in ecosomes [13]. In the context of mastitis, some studies had been performed on miRNAs. The role of microRNAs in immune function and infection rate in cattle were investigated [14]. Identification of targeted microRNAs and functional single nucleotide polymorphisms of the BOLA-DQA2 gene studied in dairy cattle [15]. Also differentially expressed microRNAs in testicular and ovarian tissues were studied in holstein cattle [16]. In another study differentially expressed microRNAs between fetal and adult backfat in cattle were studied [17]. In addition, in another study extracellular miRNAs in the follicular fluid of oocytes of cows were examined [18]. Also in a research The role of microorganisms as biochemical markers in cow's pre-pregnancy period was investigated [19]. In another study, the researchers noted the importance of microarrays and the statement that micro-RNAs play a very important role in transcriptional regulation and post-transcriptional processes of gene mutation and DNA demethylation, a global map for microRNAs and their interactions was designed. The number of reported miRNAs in the mammary glands is remarkably different (Table 1) [20]. It was discovered that the role of microorganisms in mastitis in dairy cattle was considered by researchers. In this study to uncover the genes involved in the development of mastitis, we thought to combine meta-analyzed DNA microarray data. We believed that jointly seen the interaction of miRNA could provide biologically motivated tools to manage and control mastitis. Actually we combined EST with miRNA in which EST were extracted by help of meta-analyzed DNA microarray. The prediction process of microRNA was shown in Diagram 1. Finally, important signaling pathways were identified by the microarrays involved in mastitis. The advantage of this method is that, with the identification of miRNAs involved in mastitis, it is easy to design anti marker (RNA) for the treatment of mastitis. Therefore, in the treatment of diseases or for a particular biological state, anti-micro-RNA can be used instead of extinguishing a gene, which is very difficult and in some cases impossible.

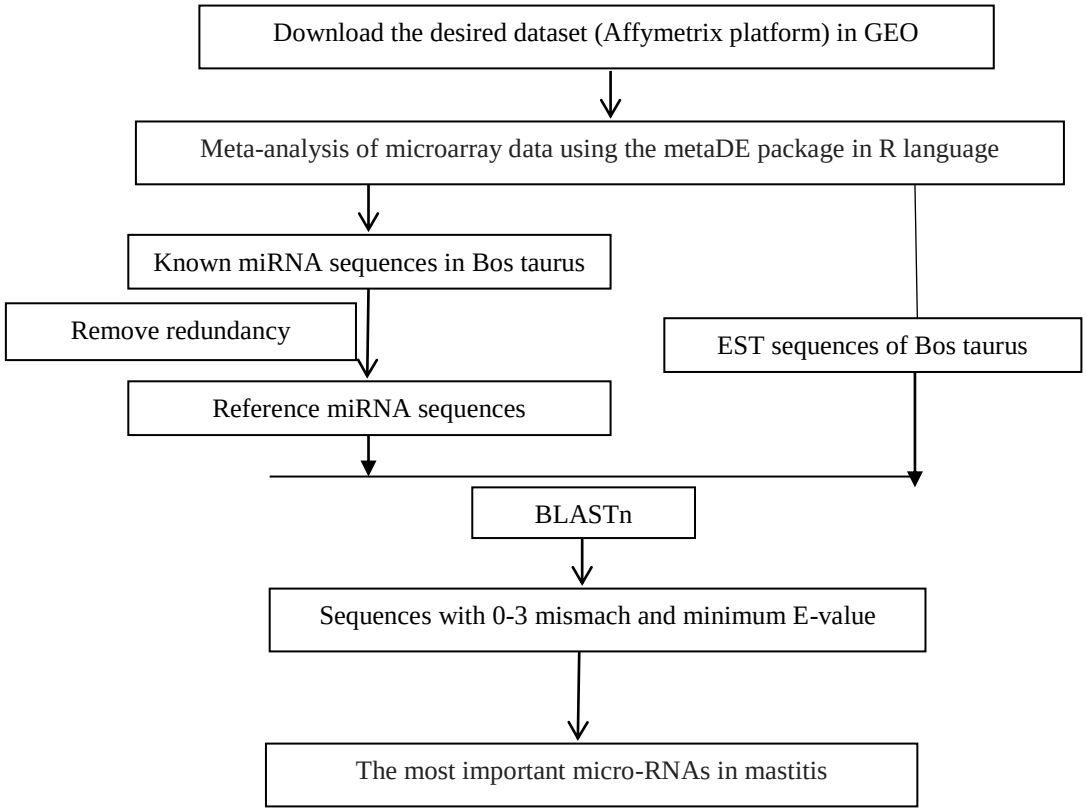

Diagram 1: Flowchart of Bos taurus miRNAs prediction in mastitis.

Table 1. Total number of miRNAs reported in mammary gland in cow, goat and sheep.

Species	Number of microRNA	References
Cow	921	[21]
Goat	441	[22]
Sheep	101	[23]

Materials and Methods

Microarray data.

The following datasets GSE15019, GSE24217, GSE24560, GSE25413 and GSE50685 were obtained from Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database. A total of 5 mastitis and 5 normal control (NC) samples were available in the GSE15019 dataset, GSE24217 contained 26 mastitis and 23 normal control (NC) samples, GSE25413 contained of 8 mastitis and 6 normal control (NC) samples, GSE50685 contained 15 mastitis and 5 normal control (NC) samples and the GSE24560 dataset included 88 samples, 5 mastitis and 5 normal control (NC) samples. The platform of these data was GPL2112 (bovine) Affymetrix Bovine Genome Array.

Microarray meta-analysis for DEGs.

The GSE15019, GSE24217, GSE24560, GSE25413 and GSE50685 were processed using http://media.affymetrix.com/support/developer/powertools/changelog/gcos-agcc/cel.html. The metaDE (https://cran.r-project.org/web/packages/MetaDE/index.html) package in R language was used to do meta-analysis. In this way, Fisher's exact test was used. Next, clustering analysis was performed to detect the distinguishing effect of metaDE on differential expression in different sample groups. The threshold for DEGs was set at a false discovery rate (FDR) of <0.01 to statistically screen out significant difference between gene expressions.

miRNA reference sets

All known miRNA sequences in various animal species including cows were obtained from miRBase (http://www.mirbase.org/). To avoid the overlapping miRNAs, the repeat sequences of miRNAs within the above species were removed. The remaining sequences were used as a reference of miRNA. The EST, and sequences of 32 genes differentially expressed genes (DEGs) were obtained from the NCBI, which were used for predict miRNAs involved in bovine mastitis.

Identification of potential miRNAs

All known miRNA sequences in various animal species including cow were obtained from miRBase (http://www.mirbase.org/ (Release 14 Sept 2010)). To avoid the overlapping miRNAs, the repeated sequences of miRNAs within the above species were removed. The remaining sequences were used as a reference of miRNA. At last, the total 1,651,501 EST sequences of Bos taurus (Bovine) were obtained from the GenBank nucleotide databases at NCBI (http:// ftp.ncbi.nlm.nih.gov; May 2016). In this study, for running a blast between EST & microRNA, Blastn program version 2.3.1 was used to predict miRNA target.

Results

As it can be seen in the above, using key terms “mastitis” and “Bos taurus" in GEO, we only selected studies that used Affymetrix bovine microarrays platform only. We found 5 appropriate studies containing 181 microarrays. In four studies, live E. coli were used in vivo, in three heat-inactivated E. coli was used on PMEC in vitro, in one studies similarly heat-inactivated S. aureus was used. The results of DNA microarray meta-analysis shown that a total of 32 genes were identified to be differentially expressed (P <0.01) across five GEO datasets (table1).

Table 2: identified differentially expressed genes (P <0.01) across five GEO datasets

Gene id	Gene symbol	P value
280828	CXCL8	1.4815e-17
280846	LTF	0.00570370370368488
280872	MX1	0.000882352941187632
280943	TNF	0.0060799999999927
281043	CCL2	8.00000000045209e-05
281212	GRO1	2.2222222150376e-05
281214	CXCL2	1.4815e-17
281250	IL1A	0.0060799999999927
281251	IL1B	1.4815e-17
281474	SAA3	1.4815e-17
281534	TLR2	0.00819374999999016
281666	CCL20	0.000166666666676085
281735	CXCL5	1.4815e-17
281860	IL1RN	0.00305000000000788
281871	ISG15	0.00969696969695945
282151	BCL2A1	0.000230769230731663
282291	NFKBIA	0.000799999999962969
282713	NFKBIZ	1.4815e-17
286849	CD40	0.00105000000002644
327712	CCL5	0.000900000000014309
407122	CSNK1D	0.00095789473686161
506415	RSAD2	0.00711612903225697
508869	RND1	0.00126666666664426
514076	CBF	0.000759999999999087

517354	CX3CL1	1.4815e-17
532569	S100A9	0.00305000000000788
513156	DAPP1	0.00229999999998045
535344	ZC3H12A	0.000109090909097074
535622	MAP3K8	0.000571428571460864
539555	MARCKS	0.00575714285713924
613667	CXCL3	1.4815e-17
616818	S100A8	0.00376799999997608

In this study a total of 946 microRNA for studied 32 genes were obtained. For running a blast between EST & microRNA, Blastn program version 2.3.1 was used to predict miRNA target. The criteria for the target gene identification were as follows: (1) three or fewer mismatched nucleotides at complementary sites between miRNA sequences and EST sequences; (2). E value cutoff of 0.069. For CCL2 gene (chemokine (C-C motif) ligand 2) was identified 31 gene target (table 3) For CBF gene (complement factor B) 26 target gene was identified. Similarly, there is also a table for other genes that put them in supplementary. For CCL5 gene (chemokine (C-C motif) ligand 5) was identified 25 gene target. For CCL20 gene (chemokine (C-C motif) ligand 20) was identified 4 gene target. For CD40 gene (CD40 molecule) was identified 80 gene target. For CXCL2 gene (chemokine (C-X-C motif) ligand 2) was identified 28 gene target. For CXCL3 gene (chemokine (C-X-C motif) ligand 3) was identified 64 gene target. For CXCL5 gene (chemokine (C-X-C motif) ligand 5) was identified 1 gene target. For CXCL8 gene (chemokine (C-X-C motif) ligand 8) not found gene target. For DAPP1 gene (dual adaptor of phosphotyrosine and 3-phosphoinositides) was identified 16 gene target. For GRO1 gene (chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) was identified 54 gene target. For IL1A gene (interleukin 1 alpha) was identified 13 gene target. For IL1B gene (interleukin 1 beta) was identified 69 gene target. For IL1RN gene (interleukin 1 receptor antagonist) was identified 40 gene target. For ISG15 gene (ISG15 ubiquitin-like modifier) was identified 99 gene target. For LTF gene (lactotransferrin) was identified 98 gene target. For MAP3K8 gene (mitogen-activated protein kinase kinase kinase 8) not found gene target. For NFKBIA gene (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) was identified 19 gene target. For NFKBIZ gene (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta) was identified 1 gene target. For RND1 gene (Rho family GTPase 1) was identified 13 gene target. For RSAD2 gene (radical S-adenosyl methionine domain containing 2) was identified 105 gene target. For S100A8 gene (S100 calcium binding protein A8) was identified 78 gene target. For S100A9 gene (S100 calcium binding protein A9) was identified 82 gene target. For SAA3 gene (serum amyloid A 3) was identified 87 gene target. For TLR4 gene (toll like receptor 2) was identified 23 gene target. For TNF gene (tumor necrosis factor) was identified 78 gene target. For ZC3H12A gene (zinc finger CCCH-type containing 12A) was identified 105 gene target. Then these EST sequences were subjected to secondary structure prediction by using MFOLD. The results obtained by MFOLD were inspected manually for determining the sequence of a miRNA precursor and appropriate stem-loop. After analyzing outputs, we identified one potential miRNA for CBF gene that fulfills all criteria described by Wang (29) The normal frequency of miRNA from EST is 0.01% (1 in 10,000 ESTs) (33) While performing a BLAST search we also set the e-value very low to find out a more significant matching region among the ESTs and reference mature animals miRNAs, thus we got only sequences with 100% similarity and few mismatches. Similarly, there is also a Diagrams for other genes that put them in supplementary files.

Table 3: Blast results between miRNA and EST in CCL2 gene

CBF	accession	E value	mismach
bta-miR-2486-5pMIMAT0012081	EE371928.1	0.53	1
bta-miR-1777a MIMAT0012032	EV706043.1	0.003	0
bta-miR-1777a MIMAT0012032	EV634897.1	0.003	0
bta-miR-1777a MIMAT0012032	EE898205.1	0.003	0
bta-miR-1777a MIMAT0012032	DY117132.1	0.003	0
bta-miR-1777a MIMAT0012032	DY113121.1	0.003	0
bta-miR-1777a MIMAT0012032	DN639096.1	0.003	0
bta-miR-1777a MIMAT0012032	DN530866.1	0.003	0
bta-miR-1777a MIMAT0012032	DN515100.1	0.003	0
bta-miR-1777a MIMAT0012032	DN275257.1	0.003	0
bta-miR-1777a MIMAT0012032	CO892262.1	0.003	0
bta-miR-1777a MIMAT0012032	CB459834.1	0.003	0
bta-miR-1777a MIMAT0012032	CB446395.1	0.003	0
bta-miR-1777a MIMAT0012032	CB438417.1	0.003	0
bta-miR-1777a MIMAT0012032	AU233350.1	0.003	0
Bta-mir-1777bMIMAT0012046	EV706043.1	0.012	0
Bta-mir-1777bMIMAT0012046	EV634897.1	0.012	0
Bta-mir-1777bMIMAT0012046	EE898205.1	0.012	0
Bta-mir-1777bMIMAT0012046	DY117132.1	0.012	0
Bta-mir-1777bMIMAT0012046	DY113121.1	0.012	1

Bta-mir-1777bMIMAT0012046	DN639096.1	0.012	0
Bta-mir-1777bMIMAT0012046	DN530866.1	0.012	1
Bta-mir-1777bMIMAT0012046	DN515100.1	0.012	0
Bta-mir-1777bMIMAT0012046	DN275257.1	0.012	0
bta-miR-2428 MIMAT0011998	EV630284.1	0.001	0
bta-miR-2428 MIMAT0011998	EH130817.1	0.001	0
bta-miR-2428 MIMAT0011998	EE914028.1	0.001	0
bta-miR-2428 MIMAT0011998	EE244861.1	0.001	0
bta-miR-2428 MIMAT0011998	DV831173.1	0.001	0
bta-miR-2428 MIMAT0011998	EE364333.1	0.001	0

KEGG pathways:

The results of 32 differently expressed genes showed that miRNAs bta-mir-1777b, bta-mir-1777a, bta-mir-2382-3p, bta-mir-2382-5p, bta-mir-671, bta-mir-2888 and bta-mir-370 have the highest frequency. These micro-RNAs have the most effect on mastitis through the four cell signaling pathways Toll-like receptor signaling pathway, pathway in cancer, Estrogen signaling pathway and Progesterone-mediated oocyte maturation (Diagrams 2-5). These miRNAs result in this pathway through cell cycle processes, DNA repair and cell proliferation, glycolysis, gluconeogenesis, and apoptosis. The signaling pathways are the mechanisms by which the cell decides on its fate and communicates with other cells and its environment [24].

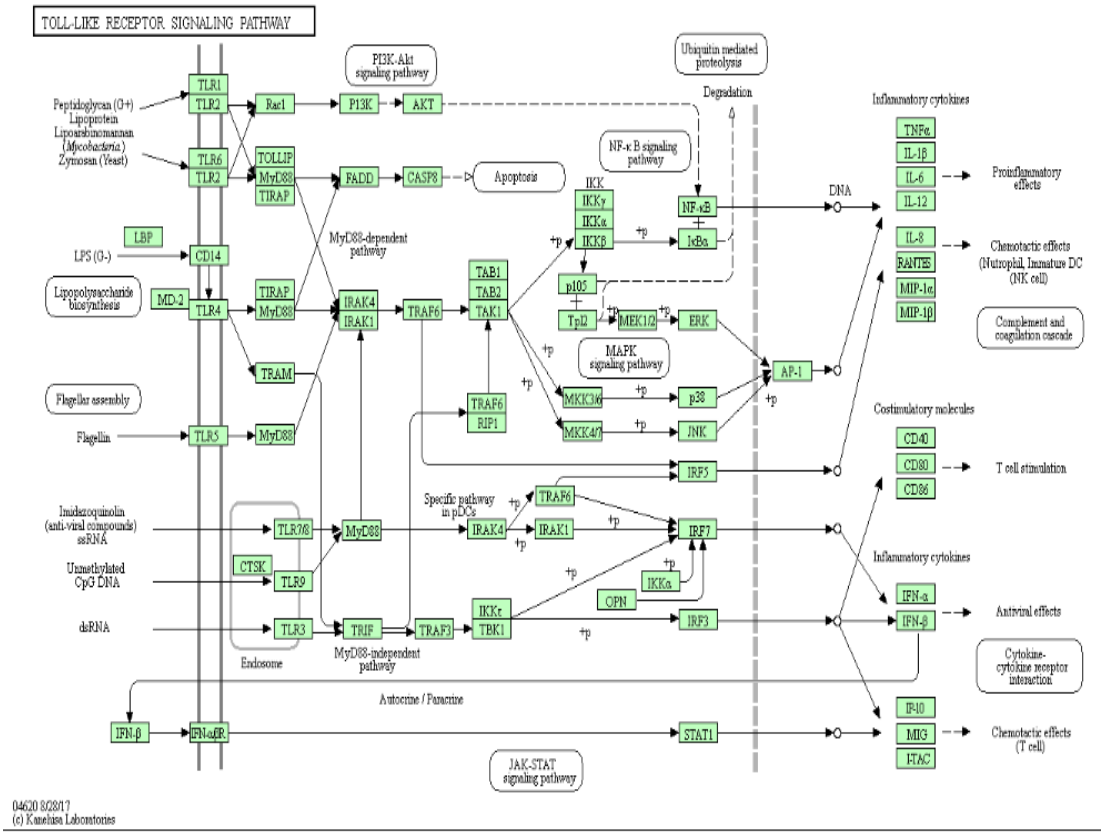

Diagram 2: KEGG pathway: TOLL-Like Receptor Signaling Pathways

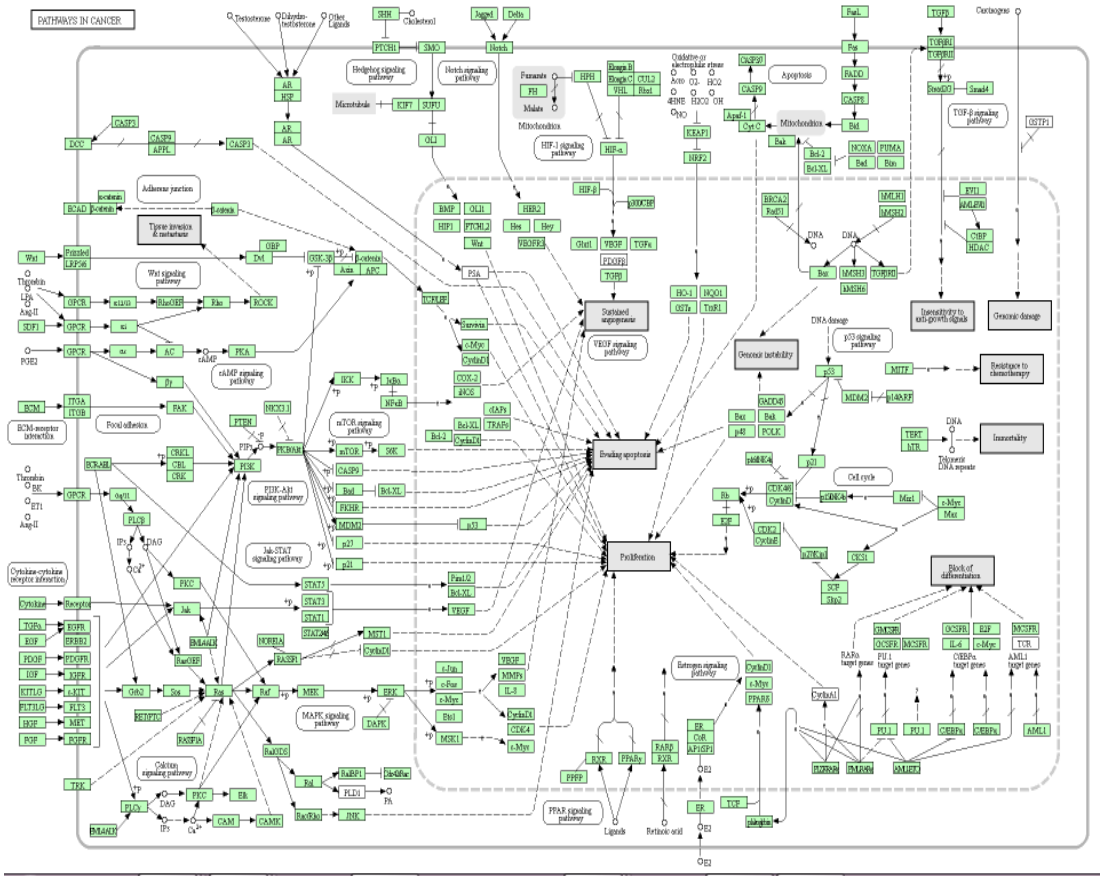

Diagram 3: KEGG Pathway: Pathway in Cancer

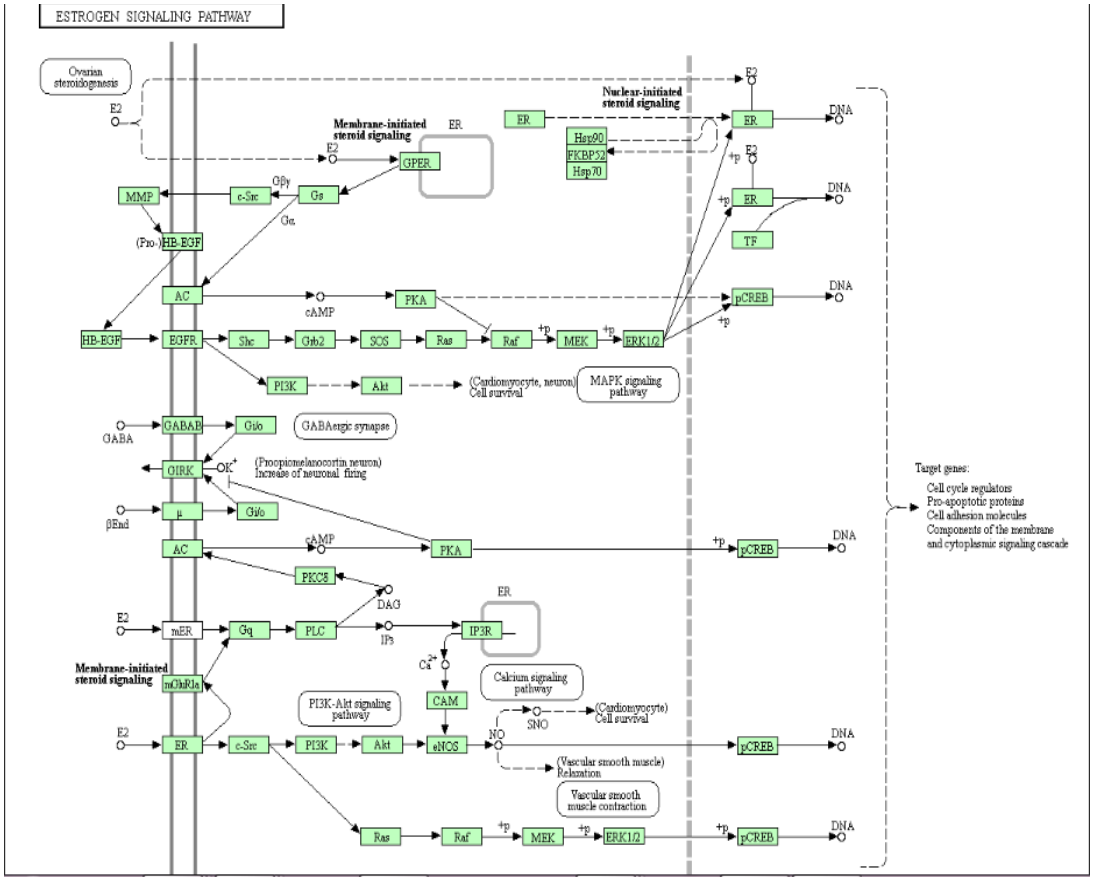

Diagram 4: KEGG Pathway: Estrogen signaling pathway

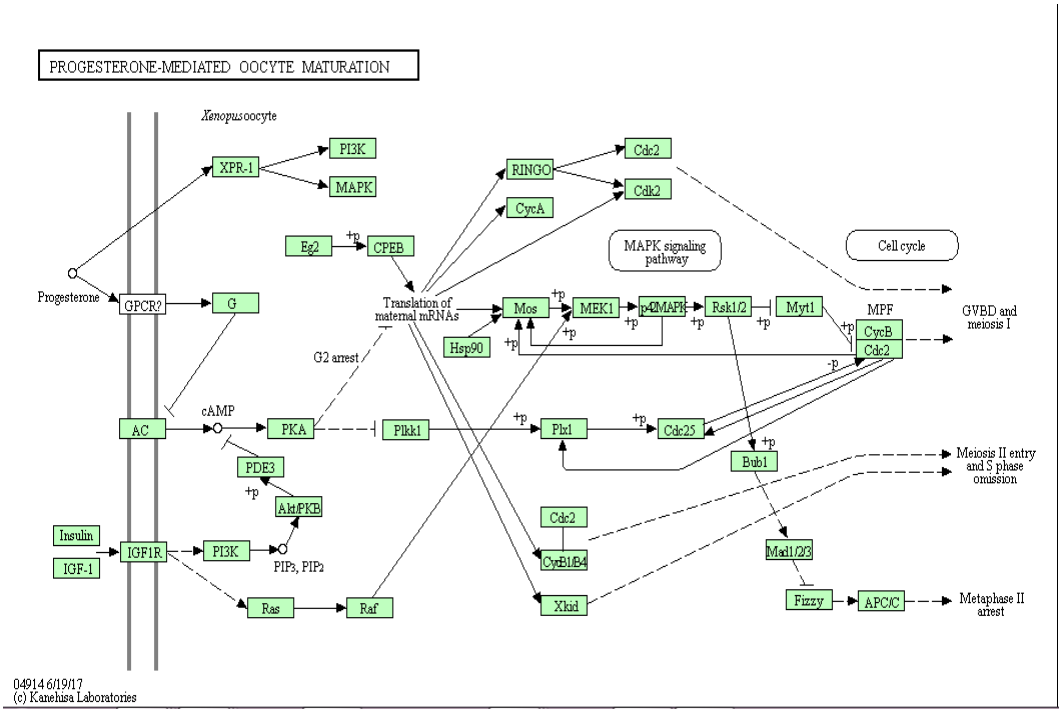

Diagram 5: KEGG Pathway: Progesterone-mediated oocyte maturation

Gene Ontology

The Gene Ontology (GO) is a bioinformatics resource that uses structured controlled vocabularies (ontologies) to describe the molecular functions or activities of a gene product, the biological processes in which a gene product is involved and the cellular components in which a gene product is located. Diagram 3 represent Gene Ontology (GO) associated with genes that are differentially expressed.

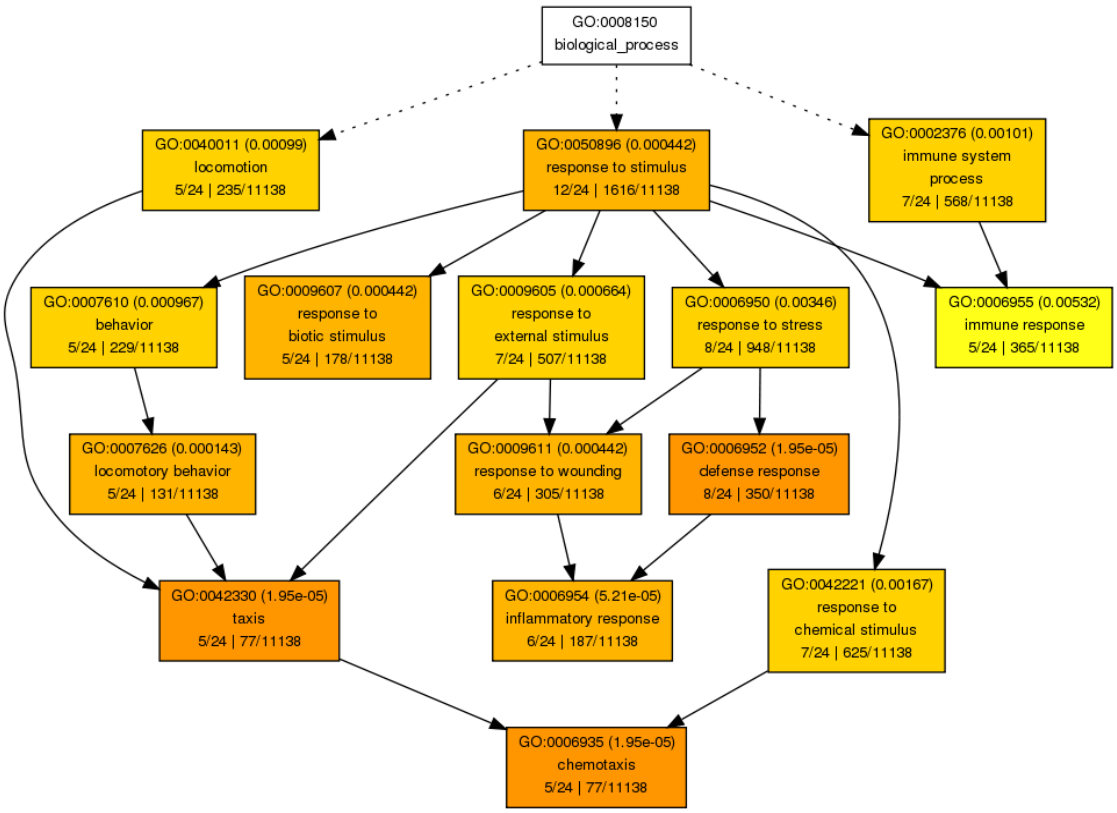

Diagram 6: Gene Ontology (GO) associated with 32 genes that are differentially expressed.

Discussion

Given that mastitis is a disease in the dairy cattle with a low heritability, the role of inheritance in this disease is low and as a result, the role of epigenetic effects in the disease is high. Therefore, in this study, we decided to study the disease from the epigenetic aspect. An important discussion here is whether these miRNAs have a role in inducing the expression of target genes or in the role of inhibitors. In diseases such as mastitis, investigating the signaling pathways of such diseases can in dairy cattle, can help manage the disease. For example, if the expression or bioavailability of the extracted signaling pathways is desirable or that it can lead to a better management of mastitis in dairy cattle, and the miRNAs examined will have an increasing role for the referred pathways, by increasing the expression of these miRNAs, it is possible to improve the expression of the pathways mentioned above and, consequently, the management of mastitis biological management in dairy cattle. Therefore, instead of looking at the massive volumes of target genes, it is possible to explore mastitis in dairy cattle with a few injectable signaling pathways and smaller number of miRNAs. This method of investigating and researching miRNAs using bioinformatics and using valid databases and finding genes that play an important role in relation to bio-candidate miRNAs is a new method in research in this category. In recent years, the use of miRNAs as biomarkers has been widespread in the treatment of many diseases. For example, in a research, the expression of genes involved in the pathway of the T cells in the cow's immune cells after the onset of Bovine Amylodotic Spongiform Encephalopathy (Bovine Amylodotic Spongiform Encephalopathy) was carried out. The effect of sponge encephalopathy on the expression of the gene in the immune cells of the cattle was studied in cattle that were treated with BASE by intracranial inoculation compared with control animals. It has been shown that among the immune pathway pathways, the genes are most affected by the activation pathways of T cell receptor T receptors [25]. In another study, KEGG's pathway analysis showed that among the studied genes, 248 genes were involved in 13 outcome sequences related to the response to pre-inflammation of the breast tissue. In addition, 13 pathways of the two important consequences were also unexpectedly identified: the natural killer cell mediated cytotoxicity pathway and the RIG-I-like receptor signaling pathway [26] In this study, it has been shown that better exploring of miRNAs can be reduce the use of genes that are of higher importance in biological pathways in breeding models, thus reducing the occurrence of mastitis. We are in an exciting time of new discoveries and a new understanding of gene regulation and epigenetic effects. Future research will improve our understanding of the role of miRNAs in health and disease and their importance as food resources.

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