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Original Article

Evaluation of the Effects of Bovine Leukemia Virus MicroRNAs on the Expression Level of TET3 and MLL2 Genes

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Abstract

Background and Aims: Bovine leukemia virus (BLV) usually infects B lymphocytes and expresses a cluster of ten microRNAs (miRNAs). The microRNAs produced by the virus in infected B lymphocytes usually constitute about 40% of all the miRNAs of the infected B-lymphocytes. The purpose of this study, bioinformatics investigation was done to identify the number of target genes of each of the BLV- miRNAs. Also, the evaluated of the expression level miRNA-B4-3p, B2-5p, and their target genes *TET3*, *MLL2*.

Materials and Methods: Each miRNA-BLV sequence was obtained from the miRBase sequence database and was then assessed by TargetScan and miRWalk software to investigate the target genes of each miRNA of the virus. In the experimental phase of the study, the infected animals were divided into two groups based on the two forms of BLV+ infection; PL form compared with AL form (Group 1) and BLV+ with normal lymph nodes compared with lymphosarcoma form (Group 2). RNA extraction was performed and cDNA synthesized for genes. qPCR was used to evaluate the expression levels of the miRNA and target genes. The expression levels of two miRNA B4-3p, B2-5p, and two genes *TET3*, *MLL2* in two groups were compared.

Results: The Fold Change (FC) values for B4-3p and B2-5p in group 1 were 22 and 67, respectively. In group 2 for B4-3p and B2-5p were 47 and 133, respectively. But there was no change in the expression level of these two gent *MLL2* and *TET3* in the two groups.

Conclusion: Despite a significant increase in expression level in B4-3p, B2-5p. there was no significant difference in *MLL2* and *TET3* expression in group 1and 2.

Keywords: BLV; miRNA; TET3; MLL2

Introduction

Enzootic bovine Leukosis (EBL) or bovine Leukosis is a viral infectious disease in cattle caused by the retrovirus bovine leukemia virus (BLV). It was first identified in 1969 under an electron microscope (1). BLV is a member of the Retroviridae family, and belongs to the genus Deltaretrovirus. It has been identified as an oncogenic retrovirus. HTLV-1,2 viruses are important viruses in the *Retroviridae* family, which show structural similarity to BLV (2). The study of retro-viruses, unlike other viruses, has particular difficulties because of the expression of reverse transcriptase (RT) and integrase enzymes, as well as the conversion of viral RNA to cDNA and the creation of provirus by the integration of virus genome within the host cell genome. Deltaretrovirus is also involved in tumorigenesis. During tumorigenesis, viral particles are not produced, and no structural proteins of the virus are transcribed. Non-structural proteins of the virus with a regulatory role are highly transcribed during tumorigenesis (3).

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Following the exposure of cattle to BLV, the virus infects lymphocytes and begins to replicate within the cells, producing a large number of viral particles within 4-8 weeks. This elicits a cellular and humoral immune response, and subsequent production of large amounts of antibodies against the viral antigens gp51 and p24, and thereby limiting the replication of the virus (4).

Deltaretrovirus infection has a long incubation period with different clinical manifestations. Almost 70% of BLV-infected animals are asymptomatic carriers of the virus and are referred to as aleukemic (AL), but the viral DNA is integrated into the host cell DNA as a provirus. Detection of the provirus and/or the virus-specific antibody confirms the diagnosis. In about 30% of infected cattle, there is a non-malignant proliferation of a specific colony of untransformed B lymphocytes that express the surface markers CD5+ and IgM+. This leads to persistent lymphocytosis (PL), a condition that can be detected by the persistent increase in peripheral blood B-lymphocytes (>10000 mm). However, this group of cattle may have a significantly increased risk of opportunistic infections due to immunological dysregulation (e.g., mastitis) (1). In less than 5% of the cases, the persistent proliferation leads to malignant B lymphocytic lymphosarcoma, often within 1 to 8 years after infection. Lymphosarcoma may occur in lymph nodes of various organs such as the liver, spleen, heart, uterus, and spine. However, the clinical symptoms associated with malignancy vary depending on the organ involved. In severe cases, mortality is very high (1, 2).

BLV carries the classical genes (gag, pro pol and env) that are required to complete the viral cycle: genesis and budding of a virion, infection of a target cell, reverse transcription and integration into the host cell chromosome. The BLV provirus also encodes a series of additional accessory genes, and miRNAs that modulate viral or cellular gene expression (Fig. 1) (2).

MiRNAs are small noncoding RNAs involved in the regulation of cellular gene expression (5). An important aspect of our study is the BLV virus miRNAs, which seem to be of great importance in BLV infections. The BLV virus contains a cluster of miRNA located between env and R3 genes with a sequence of 670 bp; within this range, five stem-loops (B1, B2, B3, B4, B5) are formed that eventually produce ten miRNAs because both strands can become mature miRNAs (15). They can target a variety of genes. In addition to affecting the expression of cellular genes, BLV miRNAs appear to inhibit the expression of their genes like herpes viruses and also prevent the formation of viral particles (6).

In the current study, the expression levels of two miRNAs (miR-B4-3p and B2-5p) in bovine infected with PL (persistence lympho-cytosis) and Lymphosarcoma were compared with controls. The miR-B4-3p is shared in a similar seed region to the eukaryotic miR-29. Therefore, its role in tumorigenesis seems to be more important (7). It has been found that the BLV miR-B2-5p shares seed identity with the host miRNA, miR-943. Although little is known regarding the function of miR-943, this micro RNA is over-expressed following the suppression of the tumor suppressor p53 activity and is highly up-regulated in the stem population of primary human mammalian epithelial cells, suggesting a possible onco-genic role of miR-943 (7).

In this study, the expression of two tumor suppressor genes, usually targeted by BLV miRNAs was also evaluated and compared between BLV infected with PL (persistence lymphocytosis) and Lymphosarcoma were compared with controls cattle. The two genes (TET3, and MLL2 (KMT2D)), which play important roles as tumor suppressors were selected for this purpose. The MLL2 gene encodes histone methyltransferase and also interacts with RNA polymerase II (8). The MLL2 protein is involved in various cellular processes such as DNA replication and maintenance of genome integrity. A decreased expression of this gene results in mutations in histones (9). The TET3 gene (ten-eleven translocations) is a member of the deoxygenase family. The enzyme. The enzyme converts 5-methyl cytosine (5 mc) to 5-hydroxymethyl-cytosine (5hmc) (10).

Table 1. Primers sequences	
Gene	**Primer sequence**
TET3	f-5'TGACTCCCTCACAACCTATCT-3' r-5'GTGCGGGACTGTATAACCTATG-3'
MLL2	f-5'GCCCAATCCTCTGTTTCCTTA-3' r-5'GGCTGTGGGCCCTATGTTATT-3'
b-actin- internal control	f-5'TCCCTGGAGAAGAGCTACGA-3' r-5'GGCAGACTTAGCCTCCAGTG-3'
BLV-B4-3p f	TCCTAGCACCACAGTCTC
BLV-B2-5p f	ATGGATGACTGAGTGTAG
bta-5s – internal control	GGAGGCTAAGCAGGG

Studies have shown that reduced TET3 function increases the incidence of myeloid leukemia and decreases genome stability. It has been hypothesized that reduced expression will lead to instability in the cell's DNA and impairment of DNA methylation (11,12).

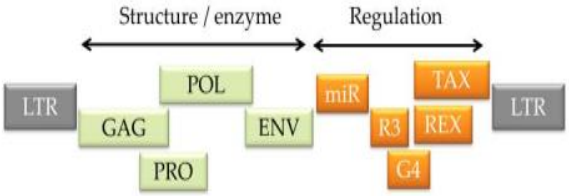


Fig. 1. Schematic structure of the bovine leukemia virus (BLV) genome (2)

Methods

In silico study: In the first stage of the study, the number and type of target genes of each miRNA were evaluated. For this purpose, the sequence of each of the miRNAs was selected and downloaded from the miRBase database (http://www.mirbase.org/) and using the software TargetScan (http://www.targetscan.org/vert_72/) and miRWalk (http://zmf.umm.uniheidelberg.de/apps/zmf/mirwalk2/) the target genes of each of the miRNAs of BLV were identified.

In vitro study: For in vitro studies, the infected cows that were BLV+ were grouped into 2; Persistent Lymphocytosis (PL) group (10 animals), and cows in the lymphosarcoma stage (> 5 years old) were considered as another group (10 animals).

peripheral blood mononuclear cells extraction (PBMCs) were taken for the evaluation of the PL group animals, and lymph nodes samples were used for evaluating the cows with lymphosarcoma. Two control groups consisting of 10 BLV+ cattle with normal lymph nodes and 10 BLV+ cattle that were AL each were also considered. To select the infected cattle in the PL group, cows with a mean lymphocyte count of more than 10,000 per ml were selected and monitored over a period of 6 months. Blood samples were diluted with PBS, and a ficoll solution was used for Lymphocytes extraction. The cattle infected with proviral DNA BLV were confirmed using the PCR method. The DNA was extracted from 100 µl of whole blood and lymph node with a reagent kit for DNA isolation from clinical samples (CinnaPure-DNA-PR881613) the manufacturer's recommendations .

Primers were designed for TET3 and MLL2 genes and B-actin was used as the internal control. Also, qPCR primers were designed for BLV-miR-B4-3p and B2-5p and the 5S gene as an internal control (Table 1).

Total RNA was extracted from tissue samples and PBMCs according to the commercial kit protocol (Bonyakhte extraction, Iran). After extracted, RNA concentration was calculated using 1.5 µg of RNA sample, and the absorbance values of the samples were determined at 260/280 nm using a NanoDrop Spectrophotometer. If the absorbance ratio was higher than 1.8, the samples were used for

cDNA synthesis. The cDNA synthesis of genes was different from microRNA. RevertAid H-minus M-MuLV kit (Fermentas, USA) was used to prepare cDNA from total RNA for gens. In this regard, 1000 ng of RNA in a volume of 20 microliters was incubated at 25 ° C for 5 min, followed by 42 ° C for 60 min, and 70 ° C for 5 min. Concentration and purity were measured and validated based on NanoDrop, as described above. To synthesize cDNA of miRNAs, polyA tail was first synthesized using five µg of total RNA according to the kit protocol, and then cDNA was synthesized using polyadenylated RNA according to the protocol (Bonyakhte extraction Kit, Iran).

In miRNA qPCR Briefly, each reaction contained 6.5 µl qPCR master mix, 1 µl specific forward and universal reverse primer, 1 µl of cDNA, and 4.5µl nuclease-free PCR grade H2O for a total reaction mixture of 13 µl. Non-template control (NTC) was included in each assay. The thermal cycling conditions were as follows: 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s, using Real-time ABI 7300 Step one Plus system, BON-miR High-Specificity miRNA qPCR Core Reagent Kit (BON2093002-Bonyakhte-Iran).

Data were then prepared to investigate the expression of genes and miRNAs Gene expression was measured by comparing the mean CT (Cycle Threshold) and FC. Gene expression levels in the infected cattle and control groups were compared using T-test. P-value < 0.05 was considered as statistically significant. Finally, the results were analyzed using Graphpad Prism.

Results

In silico studies and miRNA target genes: The present study was conducted in two phases. In the first phase, an in silico study was carried out to identify the target genes of each of the 10 miRNAs of BLV. Micro RNA sequences were downloaded from the miRbase database, and TargetScan and miRWalk were used to identify the miRNA gene targets. In the present study, sequence analysis identified 1595 genes with 3'-UTR sequences complementary to the seed region sequences in the miRNAs of BLV (Table 2).

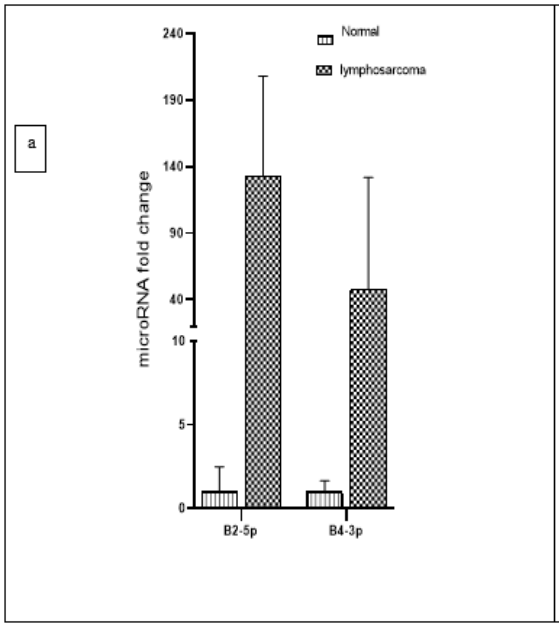
Table 2. number of target genes of each of the BLV miRNAs

BLV-miRNA	Gene Target
B1-3p	231
B1-5p	192
B2-3p	8
B2-5p	101
B3-3p	9
B3-5p	114
B4-3p	760
B4-5p	11
B5-3p	7
B5-5p	162
Total	1595

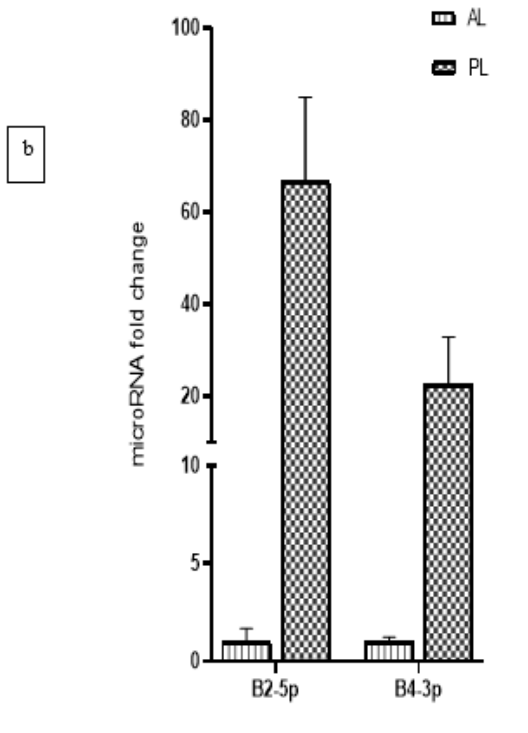
Genes that were targeted by two different miRNAs of the virus were subsequently identified. Our results demonstrate that 368 genes out of the total 1596 genes were targeted by two different miRNAs of the virus, which include important genes. Among the 368 genes, 8 genes were found to be targeted by three miRNAs (Table 3).

Table 3. Eight important intracellular genes that play an important role in intracellular signaling, along with miRNA-BLV that targets them.

Gene targets	**Ensemble-ID**	**BLV-microRNA**
TET3	ENSBTAG00000022381	B4-3p,B2-5p,B1-3p
MLL2	ENSBTAG00000014429	B1-5p,B2-5p,B3-5p
SMARCD1	ENSBTAG00000037935	B1-5p,B1-3p,B3-5p
SNX1	ENSBTAG00000002014	B1-3p,B4-3p,B5-5p
PIK3R3	ENSBTAG00000002979	B3-5p,B4-3p,B5-5p
IGF1	ENSBTAG00000011082	B1-3p,B3-5p,B5-5p
NFAT5	ENSBTAG00000013412	B1-3p,B2-5p,*B4-3p*
HIF3A	ENSBTAG00000018948	B1-5p,B2-3p,*B4-3p*



miRNA -B4-3p	
Unpaired t test with Welch's correction	lymphosarcoma vs. Normal
P value	**<0.0001**
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=5.789, df=17.41
Difference between means ± SEM	**5.565 ± 0.9613**
95% confidence interval	3.541 to 7.589
R squared (eta squared)	0.6581
miRNA – B2-5p	
Unpaired t test with Welch's correction	Lymphosarcom vs Normal
P value	**<0.0001**
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=6.936, df=14.00
Difference between means (B - A) ± SEM	7.056 ± 1.017
95% confidence interval	4.874 to 9.238
R squared (eta squared)	0.7746



miRNA-B2-5p	
Unpaired t test with Welch's correction PL vs AL	
P value	**<0.0001**
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=7.645, df=13.58
Difference between means ± SEM	6.065 ± 0.7933
95% confidence interval	4.359 to 7.771
R squared (eta squared)	0.8115
miRNA- B4-3p	
Unpaired t test with Welch's correction	PL vs AL
P value	**<0.0001**
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=7.536, df=17.59
How big is the difference?	
Difference between means ± SEM	4.498 ± 0.5969
95% confidence interval	3.242 to 5.754
R squared (eta squared)	0.7635

Fig. 2 (a, b). Comparison of expression levels of two miR-B4-3p, and B2-5p in PL and lymphosarcom animals compared with AL and normal lymph node animals. The expression of both miRNAs in the patient group (PL and lymphosarcoma) was significantly increased compared to the AL and normal lymph node animal. (c, d) In analysis Unpaired T Test with Welch's correction PL vs AL and lymphosarcoma vs normal lymph node.

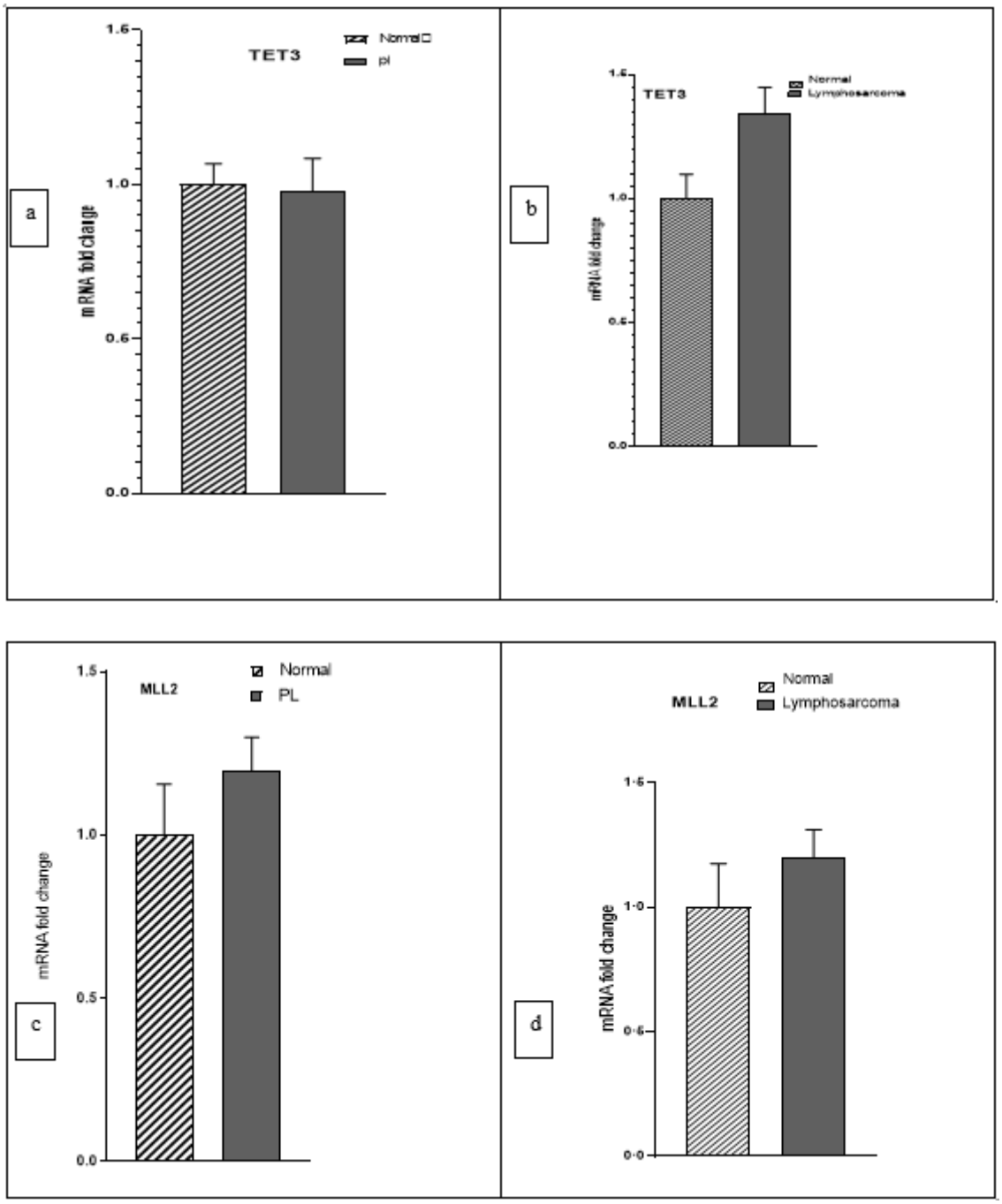


Fig. 3. (a) There was no significant difference in TET3 gene expression between PL animals and AL animals. (b) TET3 expression in lymphosarcom group not only showed no decrease but also an increased expression level compared with normal lymph node was found. (c) Comparison of MLL2 gene expression in PL animals with AL animals showed no significant difference between the two groups. (d) Comparison of MLL2 expression in normal lymph node animals with lymphosarcoma revealed no significant difference in gene expression.

After examining each of the genes in Table 3 and their intracellular signaling, the two tumor suppressor genes TET3 and MLL2, were selected for further investigation. Studies have shown that decreased expression of these genes could lead to changes in DNA methylation and histones, and eventually, tumorigenesis. Therefore, they were selected for the next stage to examine the expression (13,14). We found that these genes are targeted by three different miRNAs of BLV. Each of the high-score miRNAs targets this gene .In this study, each primer was designed using Snapgene software, as well as online IDT software (oligo dt).

Results of laboratory studies

Comparison of expression level miR-B4-3p, B2-5p and target gene TET3 in group 1 and 2: Studies on miR- B4-3p target genes demonstrated that the TET3 gene has eight conserved target loci and four non-conserved target loci at 3'_UTR for this miRNA.

Our bioinformatics studies revealed that three of the BLV miRNAs target the TET3 gene, including BLV-miR-B4-3p, B2-5p, and B1-3p, and the hypothesis that TET3 gene expression should be reduced in BLV infections was strengthened. To test this hypothesis, we chose two of the miRNAs of the virus, miR-B4-3p and B2-5p, which have high transcript levels in infected B lymphocytes to evaluate their effects on the TET3 gene (15). Analysis of the results of qPCR showed that the expression levels of BLV-miR-B4-3p and BLV-B2-5p in group 1, cattle with PL infection were significantly increased (p value $\leq$ 0.0001) compared to the AL group. The FC for B4-3p and B2-5p was 22 and 68, respectively. However, no significant difference was observed in TET3 expression between PL and AL groups. A decrease in TET3 expression was not observed contrary to our expectations. In the group 2, Lymphosarcoma compared with normal lymph node, the expression of miR-B4-3p and B2-5p increased significantly (p-value $\leq$ 0.0001), and the FC values for miR-B4-3p and B2-5p were 47 and 133, respectively. However, no significant change in TET3 gene expression was observed (Fig. 2,3).

Comparison of expression level miR-B2-5p and target gene MLL2 (KMT2D) in group 1 and 2: The MLL2 gene is of particular importance in leukemia patients, and a decrease of function leads to genomic instability and, ultimately, mutation (8).

Therefore, we investigated its expression in cows with BLV infection, groups 1 and 2. Our study in silico revealed that three of the BLV miRNAs target the MLL2 gene, including BLV-miR-B1-5p, B2-5p, and B3-5p, and the hypothesis that MLL2 gene expression should be reduced in BLV patients was strengthened. To test this hypothesis, we chose one of the miRNAs of the virus, miR-B2-5p, which has a high transcript in infected B lymphocytes (15).

Analysis of the results of qPCR showed that the expression levels of BLV-miR-B2-5p in cattle with PL infection were significantly increased (p value $\leq$ 0.0001) compared to the AL group. The FC for B2-5p was 67.

However, no significant difference was observed in MLL2 expression between PL and AL groups.

A decrease in MLL2 expression was not observed contrary to our expectations. As well as, In the group 2, compare the lymphosarcom and normal lymph node, the expression of miR-B2-5p increased signifi-cantly (p-value $\leq$ 0.0001), and the FC values for miR-B2-5p were 133. However, no signifi-cant change in MLL2 gene expression was observed (Fig 2,3).

Discussion

MiRNAs have attracted the attention of researchers in recent years. To date, 38,589 mature miRNAs have been sequenced and registered in various organisms according to the data on the latest version of the miRBase site. MiRNAs are important regulators of various biological processes: including, cellular proliferation, apoptosis, and differentiation and development through various mechanisms such as structural changes at specific genomic sites (i.e., addition, deletion, point mutations), changes in transcription level (i.e., suppression or activation), and epigenetic changes (e.g., hypermethylation or hypomethylation of the promoter, histone deacetylation and disruption of the biogenesis pathway), (5).

Because of the close association between miRNAs and such essential biological pathways, it is not surprising that alterations in miRNAs expression level can have pathogenic consequences. This is particularly evident in the development of cancers and metastases, in which miRNAs have been shown to act as tumor suppressors and oncogenes (9,16). In recent years, studies of miRNAs among pathogens (e.g., parasites, fungi, and bacteria) have been performed, and miRNAs expressions in pathogens have been confirmed, including the identification of 400 miRNAs in

various viruses, particularly DNA viruses and retroviruses (16).

DNA viruses, especially herpes viruses, adenoviruses, and papillomaviruses, are among the first and most important viruses in which miRNAs were detected, but miRNAs have also been reported in retroviruses, most notably in the simian foamy virus, Avian leucosis virus, Bovine foamy virus, and Bovine leucosis virus. Each of these viruses has one or more miRNAs that play a key role in the pathogenesis of diseases and tumorigenesis (18,11). Other viruses such as HIV, HAV, West Nile virus, Ebola virus, and Dengue virus have also been found to have non-coding RNAs that are involved in the regulation of their target Due to the importance of retroviruses in cancer, extensive studies have been started on these viruses (18). Micro RNA expression was first reported in BLV retroviruses in 2012 (7).

The zoonotic nature of BLV infections has been the focus of attention in recent years. It has been reported that up to 70% of infected cows are asymptomatic during early infection. This could increase the risk of infection spread as their meat and milk may be unconsciously consumed by humans. Interestingly, the mammary tissue is among the organs in which the virus is actively replicating. Thus, the risk of contamination of milk in asymptomatic animals is relatively high. Due to the integration of this virus into the host cell genome, the virus may induce cellular malignancy or may have its effects many years later or in subsequent generations (19). For this reason, in recent years, a group of researchers has begun investigations into the effects of the viral genome integration into the host genome. One of these studies was conducted in 2015 by Buehring et al. on 49 women who have breast cancer. They identified fragments of BLV genome in 59% of these patients. However, breast tissue examination of healthy subjects also confirmed the presence of BLV virus (29%) (20). In addition, the study continued in 2019, the same researchers reported that the DNA of BLV virus was detectable in 38% of blood samples obtained from 95 female donors. This reinforces the zoonosis and food-born potential of the virus (21). In Iran, one study reported the presence of tax (30%) and gag (8%) genes of the virus in humans by evaluating 200 blood samples. Although the subjects did not have breast cancer, 16% of them were BLV positive (22).

Another important issue is the high similarity of the BLV virus to the human HTLV-1 virus. Both viruses belong to the genus Deltaretrovirus and are very similar in genomic structure and pathogenicity. The major difference is between the env gene and the 3'-UTR gene. In the region called px, the R3, G4 gene of BLV virus is located, whereas the P12, P13, and P30 genes of HTLV-1 virus are located at this site (23). The expression of HBZ antisense occurs in HTLV-1, but its transcript is absent in BLV, whereas AS-1 and AS-2 antisense transcripts and miRNA clusters are transcribed in BLV, with a similar function to the HBZ antisense of BLV (24).

An in-depth understanding of the pathogenesis of the BLV virus may be useful in increasing our understanding of HTLV-1 and for providing novel therapeutic strategies for both diseases (23).

A set of viral regulatory genes along with miRNAs are transcribed in infected cells. An increasing body of evidence suggests that virus tumorigenesis continues even with the deletion of the tax gene, indicating that, contrary to other studies, this gene is not a major factor in virus tumorigenesis, and thus other factors such as viral miRNAs may play a more important role in this process (24).

Deep sequencing studies have shown that the transcript levels of the miRNAs of this virus are greatly increased in BLV-infected B cells, which is in line with the results of our reaction qPCR sample evaluation. In these studies, the percentage of virus-encoded miRNAs accounted for about 40% of the total miRNAs in infected lymphocytes (25).

The miR-B4-3p targeted many genes than the other BLV miRNAs. Most of these genes have a tumor suppressor role or affect apoptosis. Studies have shown that the number of B4-3p transcripts during BLV infection is much higher than the other transcripts. Of the total number of transcripts associated with BLV miRNAs, approximately 70% are linked to

miR-B4-3p. Most B4-3p-related transcripts are found among all the viral and cellular miRNAs present in infected B lymphocytes (25).

In our study, there was also a significant increase in B4-3p expression in PL samples as compared to AL and lymphosarcoma samples as compared to normal lymph node samples. (Fig 2). MiR-B2-5p significantly increased, even more than B4-3p, in Lymphosarcoma and PL infected animals (Fig 2,3). In the advanced stages of the disease, a higher number of miRNA transcripts are inversely linked to the expression of each of the target genes of the related micro RNA. Another important point is that five of the BLV virus miRNAs (B4-3p, B2-5p, B5-5p, B1-3p, B3-3p, B5-3p) are among the first 15 miRNA of the cell to highly express during infections. Even the expression level of miR-B4-3p is higher than that of miR-17-92, a cluster of cellular miRNAs that increases in leukemia (25).

Regarding miR-B4-3p, the similarity of its seed region to the miR-29 in humans and cows should be taken into consideration, and it is possible that miR-29 targets are identical to the targets of the viral miR-B4-3p (Table 4).

Table 4 Comparison of miR-29 sequence in a cow with miR-B4-3p in BLV virus

>blv-miR-B4-3p MIMAT0025864	UAGCACCACAGUCUCUGCGCCUUU
>bta-miR-29a MIMAT0003518	CUAGCACCAUCUGAAAUCGGUUA

This may lead to a dysfunction of miR-29 (26). One of the important reasons for the diversity of function of miR-29 is its many targets. The function of miR-29 may be different based on the tissue because different studies have suggested its role as both an oncogene and tumor suppressor gene (27). For example, in human AML patients, its expression results in increased apoptosis by targeting MCL-1, CCND2, Akt2, and C MYC genes (28). It also inhibits apoptosis in Chronic myelogenous leukemia (CML) and Acute lymphoblastic leukemia (ALL) patients by inhibiting BCR/ABL1, ABL1, and BCL11B Tcl1 genes. However, it leads to reduced DNA methylation, mutation, and tumorigenesis by inhibiting the expression of Sp1, DNMT3A, and DNMT3B genes in Acute myeloid leukemia (AML) patients (27).

It seems that miRNAs of BLV, especially B4-3p, are important factors in leukemia pathogenesis in infected cows, in addition to the tax gene (12). One of the major genes targeted by miR-B4-3p is the TET3 gene with eight conserved loci at the 3'-UTR of this gene (three 8mer positions and five 7mer positions), which is targeted by a high score. Similar studies have been done on BLV miRNA target genes. In one study, reduced expression of FOS, GZMA, and PPT1 genes in Lympho-sarcoma specimens was confirmed. These genes have been implicated in the processes of apoptosis and immune response (26).

Another study demonstrated that the expression level of IgM antibodies in BLV positive samples was significantly decreased compared to control samples, due to the targeting of genes involved in the transcription of genes related to immune response. Their expression decreased by targeting IGj, BLMP1, BCL6, and PAX5 genes, leading to a decrease in IgM production (27).

Conclusion

In this study highlighted the importance of the BLV miRNAs. Our study results indicate that several genes may be targeted by each of the BLV- miRNAs, making it necessary to further evaluate the effects of these miRNAs on their target genes. As well as, we found that the expression levels of miR-B4-3p and B2-5p were significantly increased in cattle with PL and lymphosarcoma in group 1 and 2 ($P<0.05$). Thus, that despite the lack of expression of virus structural proteins and the termination of viral particle production, miRNAs are still expressed. In this study, we emphasized the significance of miR-B4-3p and miR-B2-5p and their possible role in the pathogenesis and tumorigenesis of the virus. We hypothesize that the regulation of tumor suppressor genes in the cell is influenced by the BLV-miRNAs, and the inhibition or decreased expression of these genes is associated with the tumori-

genesis of the virus. Although, in insilico study TET3 and MLL2 genes were targeted by three different miRNAs of the virus, But, no significant change was observed in their expression levels in the PL (group 1) and Lymphosarcoma (groups 2). Therefore, these genes may not be considered as agents in tumorigenesis.

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Conflict of interest

The authors declare no conflict of interest.

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