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Investigation of the target genes of BLV miRNAs and the expression levels of miR-B4-3p and miR-B2-5p in cattle infected with Bovine Leukemia Virus

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Abstract

Bovine Leukemia Virus (BLV) is an oncogenic retrovirus of the genus Deltaretrovirus. The genome of BLV encodes a cluster of 10 mature microRNAs (miRNAs). Considering the importance of miRNAs in regulating gene expression, it seems that each of the miRNAs of BLV plays a vital role in the process of pathogenesis and tumorigenesis of the virus. First, sequences of each of the miRNAs of BLV were selected and downloaded from the miRBase database. The sequences were then investigated using TargetScan and miRWalk to identify target genes of each of the mature miRNAs of the virus. Second, the expression levels of the two miRNAs with the highest number of target genes in B lymphocytes and lymphoid tissues were evaluated using qPCR and were compared between cattle with different forms of BLV infection: PL form was compared to aleukemic (AL) form (Group 1) and BLV+ with normal lymph nodes were compared to lymphosarcoma form (Group 2). We identified a total of 1595 target genes of the micro RNAs. The miRNAs with the highest target genes included miR-B4-3p with 760 and B2-5p with 102 target genes. In the second phase, miRNA expression in BLV-infected animals was investigated. The Fold Change (FC) values for miR-B4-3p and miR-B2-5p in group 1 were 22 and 67, respectively. In the second group, the FCs for miR-B4-3p and miR-B2-5p were 47 and 133, respectively. The expression was significantly higher in persistent lymphocytosis (PL) cattle in group one and lymphosarcoma cattle in group two.

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Introduction

The Bovine Leukemia Virus (BLV) is classified as a Deltaretrovirus belonging to the family Retroviridae. An oncogenic retrovirus causes Enzootic Bovine Leukosis (EBL).[1] The Deltaretrovirus has attracted the attention of many researchers in recent years. Enzootic Bovine Leukosis is of great importance due to its economic impact on the meat and dairy industry, and due to its zoonotic potential, it poses a threat to human health.[2] The BLV is widely distributed throughout the world, with a prevalence of 30.00 to 90.00% in most countries.[3] Even though it has been eradicated from Western Europe, BLV infections continue to be a public health concern in many countries.[4] In the United States, the estimated infection rates are about 83.00% among dairy cattle and 34.00% among beef cattle.[5] A high infection rate has also been reported in most Middle East countries. For example, in Iran, the estimated infection rate is 22.00%. Given the diverse transmission routes of the virus and its capacity to integrate its Provirus into the host genome, and the uncertainties regarding the process of its pathogenesis and tumorigenesis, effective preventive and treatment measures are needed to reduce the risks of the disease. However, there is still no effective vaccine or medication for preventing and treating the diseases.[6]

In recent years, BLV has been linked with breast cancer in women, which is particularly worrying given the zoonotic potential of the virus. The breast cancer is also seen in men, however, it is the most common cancer in women. Globally, more than 1.70 million new breast cancer cases in women are being diagnosed each year.[7] In a research study conducted in 2015 in the United States among 49 women with breast cancer, pathological analysis of the cancer tissues detected fragments of the BLV genome in 59.00% of the samples.[8] In a similar study

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of 172 breast cancer cases in Iran, 30.00% were positive for the tax, and 8.00% were positive for the gag gene of BLV. These findings reaffirm the importance of the virus and the need for extensive research toward reducing its prevalence in livestock worldwide.[9] It is worth mentioning that other Deltaretroviruses, including the Human T Lymphocytic Viruses (HTLV) 1 and 2 are structurally similar to BLV, and both viruses have similar pathogenicity. The HTLV-1 is widespread and every year it affects about 20 million people worldwide. Therefore, research studies towards identifying an association and differences between BLV and HTLV-1 are necessary to accurately diagnose human infections due to HTLV-1.[10] Three forms of BLV infection have been identified: Asymptomatic or aleukemic (AL) form, persistent lymphocytosis (PL), and lymphosarcoma. The Aleukemic form (asymptomatic) accounts for 70.00% of the cases. The infected cattle are serologically positive for antibodies against gp51 and P24 proteins in this form, however, they do not exhibit any clinical symptoms The aleukemic cattle do not play a role in transmitting and spreading the disease.[11] About 30.00% of BLV infections lead to persistent lymphocytosis in infected cattle. There is a persistent increase in the number of peripheral blood lymphocytes to more than 10,000 per mL in this form. This group of cattle plays an important role in transmitting the disease both horizontally and vertically. The lymphosarcoma form, the advanced form of the disease, occurs in 1.00 to 5.00% of BLV-infected animals. There is a malignant proliferation of B lymphocytes in this form leading to lymphosarcoma. Lymph node enlargement may involve various organs such as the liver and spleen. Lymphosarcoma mostly occurs in cattle aged over five years.[12] Weight loss, decrease in milk yield and opportunistic infections may lead to premature culling of the infected animals.[2]

In 1998, researchers discovered new regulatory factors in cells, later called microRNAs (miRNAs). Eleven miRNAs are a group of short-chain non-coding RNAs with a length of 18 to 24 nucleotides. Due to their major impact on gene expression, they are described as negative regulators of mRNA expression.[13] Since 1998, miRNAs have gained the attention of researchers and they have been studied for their possible role in the pathogenesis and virulence of microbes. Their role in the pathogenesis of human viruses was confirmed in Herpesvirus infections. It is now known that miRNAs play a major role in Herpesvirus latency.[14]

In 2012, researchers discovered a BLV genomic region that encodes a cluster of 10 mature miRNA in the 3 ' end of the env gene. This genomic region encodes five stem-loop hairpins with a total length of 670 nucleotides. The stem-loop hairpin structures are transcribed into a cluster of ten mature miRNAs.[15] The BLV-miRNAs accounted for about 40.00% of the total miRNAs transcribed in infected B lymphocytes and five of these virus-encoded miRNAs were among 15 miRNAs with the highest number of transcripts in the infected B lymphocytes.[16] Also, in a previous study, miR-B4-3p constituted 62.00% and miR-B2-5p 15.00% of the total BLV-miRNAs in infected cells (Table 1).[15,16] The findings of these studies paved the way for addressing ambiguities surrounding the pathogenesis of the bovine leukemic virus. Determining the importance of each of the miRNAs, cellular processes affecting them and the role of each microRNA in the expression of host genes are necessary for understanding the pathogenesis of this virus. Also, determining whether the expression of these miRNAs is associated with the pathogenicity of the virus and whether their expressions change at different stages of the disease are necessary. Knowledge in these areas can be useful in improving our understanding of the tumorigenesis of the virus and, prevention and treatment strategies for the disease. Notwithstanding, there are still limited studies in this regard.

Table 1. The number of target genes of each BLV miRNAs. In high-throughput sequencing (HTS) column, the copy number (number of transcripts) of each miRNA in infected B lymphocyte and in the HTS read (%) column, the amount of the total BLV miRNAs accounted for by each miRNA are shown, respectively.

BLV-miRNAs	Gene Target	HTS read mean[15]	HTS read (%)[16]
B1-3p	231	97,840	5.63
B1-5p	192	220	0.01
B2-3p	8	11,748	0.68
B2-5p	101	266,095	15.30
B3-3p	9	74,677	4.29
B3-5p	114	7,462	0.43
B4-3p	760	1,086,331	62.47
B4-5p	11	4,782	0.28
B5-3p	7	55,399	3.19
B5-5p	162	134,381	7.72
Total	1595	1,738,935	100

To detect BLV+, targeting tax gene of the BLV genome region coding for the oncogenic protein responsible for malignant transformation by polymerase chain reaction (PCR) amplification was aimed. Because BLV Tax can cooperate with the Ha-ras oncogene to transform cells in culture and form tumors in immunodeficient mice. Also, Tax causes the translocation of NF kB resulting in the activation of gene pathways that promotes lymphocyte proliferation or alter cell survival and causes the accumulation of cellular genetic defects, possibly leading to leukemia.[17] In deltaretroviral genomes, tax is rarely deleted during disease progression, as are the genome regions related to viral replication (gag, pol, env), and tax has the most highly conserved sequence of any deltaretroviral genome region.[9,18]

The present study was aimed to (1) evaluate the target genes of each of the BLV-miRNAs, and (2) to compare the expression levels of the miRNAs with the highest number of target genes in BLV+ cattle. In the first phase, *in silico* studies were done to identify the number of target genes

of each of the BLV miRNAs. TargetScan and miRWalk were used for this purpose. In the second phase of the study, the expression levels of the two most important miRNAs (B4-3p and B2-5p) were evaluated in BLV+ cattle. The results of this study will be useful in subsequent studies aimed to investigate the effects of these miRNAs on the expression levels of their target genes in in-vivo laboratory conditions.

Materials and Methods

***In silico* studies.** 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 Target-Scan Software (http://www.targetscan.org/vert_72/) and miRWalk (http://zmf.umm.uniheidelberg.de/apps/zmf/m irwalk2/), the target gens of each of the miRNAs of BLV were identified. The results of the *in silico* studies are listed in Table 1.

***In vitro* studies.** After evaluating the miRNA gene targets in phase one, two miRNAs with the highest number of target genes and miRNA copy number in infected B lymphocytes were selected for further studies in the second phase of the study. The expression levels of these two miRNAs were evaluated and compared between BLV+ cattle with different forms of the disease: The levels of expression of the miRNAs in peripheral blood lymphocytes were evaluated and compared between serologically positive asymptomatic (AL) cattle and cattle with PL form of the disease (first group). The expression levels of the miRNAs in lymphoid tissues were also evaluated and compared between BLV+ cattle with lymphosarcoma and BLV+ cattle with normal lymph nodes (second group).

Sample preparation. In the first group, samples were taken from cattle suspected of having PL form and aleukemic form of the disease, and the samples were subsequently confirmed as BLV+ by PCR assay. Cattle in this group were monitored for six months in a commercial dairy farm in Tehran. During this period, two peripheral blood mononuclear cells isolation (PBMCs) were taken at an interval of three months for complete blood count and differential count tests, and cattle with total lymphocyte count more than 10,000 per milliliter on each occasion were suspected of having PL form of the disease and were selected for PCR confirmation of their BLV+ status. At the same time, cattle with normal total lymphocyte count on each occasion were suspected of having the aleukemic form of the disease.

PBMCs isolation. Lymphocytes were isolated from the PBMCs samples using Ficoll's solution (Sigma-Aldrich, Delhi, India). Briefly, anticoagulated blood was centrifuged at 800 *g* for 30 min, and the top layer containing plasma was removed. The remaining blood was diluted with an equal volume of 1X, pH 7.40 phosphate-buffered saline (PBS; Sinaclon, Tehran, Iran), containing 0.05 M ethylene-diaminetetraacetic acid (EDTA; ATR-MED, Tehran, Iran). 12.50 mL of diluted blood was layered over 25.00 mL of the Ficoll. Gradients were centrifuged at 400 *g* for 30 min at room temperature in a swinging-bucket rotor without the brake applied. The PBMC interface was carefully removed by pipetting and washed with PBS-EDTA by centrifugation at 250 *g* for 10 min, then transferred to appropriate microtubes.

Lymph node specimens preparation. In the second group, specimens were prepared from lymphoid tissues from old cattle suspected of having BLV lymphosarcoma and cattle with normal lymph nodes suspected of having BLV infection. Animals in this group were also monitored for six months in an abattoir in Tehran, and animals were sacrificed and lymph node specimens were prepared. Finally, a total number of 100 samples from cattle in group one (n = 50) and group two (n = 50) were taken and analyzed for BLV+ status using PCR assay.

PCR and selection of BLV+ samples. According to the manufacturer's protocol, DNA was extracted from the samples using CinnaGen PCR kit (CinnaPure-DNA-PR88-1613; CinnaGen, Tehran, Iran). Two grams of lymphoid tissues from the cattle in group two or to 100 µL of the buffy coat of peripheral blood samples from group one was suspended in 400 µL of lysis buffer, and the mixture was vortexed for 15 to 20 sec. This step yielded a homogenous mixture. Next, 300 µL of a precipitation solution were added to the mixture and were gently mixed by a circular motion for 3 to 5 sec. The mixture was then incubated at 20.00 ˚C for 20 min. Subsequently, the resulting solution was centrifuged at 12,000 rpm for 10 min and the supernatant was gently discarded by placing the microtube upside down on a paper towel. Briefly, 1.00 mL of washing buffer was added to the sediment and mixed gently by rotating the tube for 3 to 5 sec. The mixture was then centrifuged at 12,000 rpm for 5 min, and the washing buffer was gently and completely discarded. The sediment was subsequently incubated at 65.00 ˚C for 5 min. The sediment was dissolved in 30.00 µL of TE buffer in the next step. The mixture was then incubated at 65.00 ˚C for 5 min. with gentle shaking. The mixture was centrifuged again at 12,000 rpm for 30 sec following incubation. This step yielded a supernatant containing pure DNA. DNA concentration was measured by spectrophotometry. In the next step, the primers used for PCR were designated based on the tax of BLV using GenRunner and CLC sequence viewer Software (version 6.0; QIAGEN, Aarhus, Denmark). The *BLV-tax gene* Gen Bank accession *#EF600696* primer F-5'-ATGTCACCA TCGATGCCTGG-3' R-5'-CATCGGCGGTCC AGT TGATA-3'and B-actin primer F-5'-TCCCTGGAGAAGA GCTACGA-3' R-5'-GGCAGACTTAGCCTCCAGTG-3', and BLV+ samples were detected.

Total RNA extraction from BLV+ samples. For RNA extraction, briefly, 1.00 mL of cold RNX-Plus (Sinaclon RNX Plus-EX6101; CinnaGen, Tehran, Iran) solution was added to a 2.00 mL vial containing lymphocytes harvested from 3.00 mL of peripheral blood samples from cattle in group one (PL or AL cattle) or to 100 mg of homogenized lymphoid tissue from cattle in group two (lymphosarcoma or cattle with normal lymph nodes). After vortexing for 5 to 10 sec, the vial was incubated at room temperature for 5 min, and 200 µL of chloroform (Merck, Darmstadt, Germany) was added. The final mixture was gently vortexed for 15 sec and then incubated on ice for 5 min. Subsequently, the mixture was centrifuged for 15 to 20 min at 12,000 rpm at 4.00 ˚C. The supernatant was removed, 200 µL was transferred into a new 1.50 mL vial, and an equivalent volume of isopropanol (Merck) was added. The solution was gently mixed and then incubated on ice for 15 min. The mixture was then centrifuged for 15 to 20 min at 12,000 rpm at 4.00 ˚C. The upper phase was discarded and 1.00 mL of 75.00% ethanol (Merck) was added to the pellet. The pellet was centrifuged again for 5 min at 10,000 rpm at 4.00 ˚C. The upper phase was discarded again and the pellet was air-dried at room temperature for 15 min. The RNA pellet was dissolved in 30.00 µL of deionized water and then incubated at 65.00 ˚C for 5 min. These steps yielded total RNA samples for the synthesis of cDNA. Total RNA sample (25.00 µg) was used to assess the purity of the RNA samples. Purity was assessed by determining the absorbance of the total RNA sample at a wavelength of 260/280 and where the absorbance ratio was greater than 1/8, the sample was selected for the synthesis of cDNA.

Polyadenylation and cDNA synthesis and qPCR. The reagent was mixed according to the kit protocol and the polyadenylation reaction was incubated at 37.00 ˚C for 30min followed by heat inactivation at 65.00 ˚C for 20 min. This step yielded polyadenylated miRNA for the synthesis of cDNA. The cDNA was synthesized immediately after polyadenylation. The cDNA synthesis reaction was as follows: 10.00 µL of polyadenylated miRNA was mixed with 1.00 µL of BON-RT adaptor primer (10.00 µM) in tubes and RNase-DNase free water was added to reach a volume of 13.00 µL. Next, the tubes were closed with a cap and placed on a thermocycler for 5 min at 75.00 ˚C. The tubes were immediately placed on ice and the reagent was mixed according to the kit protocol. Next, the tubes were placed on a thermocycler again, at 25.00 ˚C for 10 min, followed by 42.00 ˚C for 60 min and then at 70.00 ˚C for 10 min. These steps yielded miR-cDNA (BON miR High Sensitivity miRNA 1st Strand cDNA Synthesis kit-BON 209001; Bonyakhteh, Tehran, Iran). In the next step, using qPCR ABI 7300 Step one Plus system, BON-miR High-Specificity miRNA qPCR Core Reagent Kit (BON-2093002; Bonyakhteh) according to the manufacturer's instruction, specific forward primer and 5S primer as an internal control (miRNA-BLV-B4-3p forward primer TCCTAGCACC ACAGTCTC, miRNA-BLV-B2-5p forward primer ATGGATG ACTGAGTGTAG Internal control: bta-5s forward primer GGAGGCTAAGCAGGG), qPCR was performed for the miRNA samples based on the temperature cycling protocol in 95.00 ˚C for 1 min, 95.00 ˚C for 15 sec, 60.00 ˚C for 30 sec.

Statistical Analysis. The data were analyzed using GraphPad Prism Software (version 9.3.1; GraphPad Software Inc., San Diego, USA) and -ΔCT and Fold Change (FC) were used for data comparison. The *t*-test was used to determine statistical significance. The $p < 0.05$ were considered statistically significant.

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 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 1). Since miRNAs negatively regulate mRNA expression, it was predicted that these gene targets would not be expressed in infected cells. Most importantly, some of these gene targets played an important role in the apoptosis pathway and tumor suppression. The activities of multiple viral miRNAs on a single gene target could repress the expression of that gene in the infected cell. Gene sequence analysis in our study also showed that two different miRNAs of BLV targeted 316 genes. Thus, there was a higher chance that the expression of these genes would be negatively regulated in the infected cells. Also, in the present study, we observed that eight genes were targeted by three different miRNAs of the virus, among which there were important genes. It appears that careful investigation of the expression of these genes was necessary, as most likely, their expression would be influenced by the viral miRNAs in the infected cells (Table 2). The main purpose of the first phase of our study was to identify miRNAs with the highest gene targets and miRNA copy number in the infected B lymphocytes. Sequence analysis showed that miR-B4-3p had the highest number of target genes followed by miR-B1-3p, B5-5p, B3-5p, and B2-3p (Table 1). A previous study evaluated copy numbers of the miRNAs in the infected B lymphocytes. B4-3p accounted for 65.00% of the total BLV miRNA transcripts, followed by B2-5p (15.00%). Due to its high expression in infected cells, miR-B2-5p was selected as the second most important miRNA for further studies. By investigating the target genes of these two miRNAs, it was found that important genes that played a role in tumor suppression,

such as TP53 and PTEN, were targeted, and it was predicted that the expression of these genes in the infected B lymphocytes would be repressed. Therefore, miR-B4-3p and miR-B2-5p were considered the most important BLV-miRNAs and were selected to evaluate their expression in BLV+ cattle.

Results of laboratory studies. In the second phase of our study, the expression levels of the two selected BLV miRNAs were evaluated in infected cattle. First, 100 samples (PBMCs and lymphoid tissues) were collected from cattle suspected of having BLV infection and were evaluated for their BLV+ status using PCR. PCR studies identified 40 samples as BLV+: 20 PBMCs prepared from cattle in a commercial dairy farm in Tehran (group one) and 20 lymphoid tissue specimens prepared from cattle in an abattoir in Tehran (group two). For the 20 BLV+ blood samples, 10 samples were collected from cattle suspected of having PL cattle and 10 samples from cattle suspected of having the AL form. Also, for the 20 BLV+ lymphoid tissue specimens of group two, 10 specimens were collected from cattle suspected of having lymphosarcoma and 10 specimens with normal lymph nodes.

Table 2. Eight intracellular genes that play an important role in intracellular signaling, along with BLV-miRNA that targets them.

Target gene	BLV-microRNA	Ensemble-ID	Summary-gene target
TET3	B4-3p,B2-5p,B1-3p	ENSBTAG00000022381	Members of the ten-eleven translocation (TET) gene family, including TET3, play a role in the DNA methylation process.[19]
MLL2 (KMT2D)	B1-5p,B2-5p,B3-5p	ENSBTAG00000014429	The protein encoded by this gene is a histone methyltransferase that methylates the Lys-4 position of histone H3. The encoded protein is part of a large protein complex called ASCOM, which has been shown to be a transcriptional regulator of the beta-globin and estrogen receptor genes.[20]
SMARCD1	B1-5p,B1-3p,B3-5p	ENSBTAG00000037935	The protein encoded by this gene is a member of the SWI/SNF family of proteins, whose members display helicase and ATPase activities and which are thought to regulate transcription of certain genes by altering the chromatin structure around those genes. The encoded protein is part of the large ATP-dependent chromatin remodeling complex SNF/SWI.[21]
SNX1	B1-3p,B4-3p,B5-5p	ENSBTAG00000002014	This gene encodes a member of the sorting nexin family. Members of this family contain a phox (PX) domain, a phosphoinositide binding domain, and are involved in intracellular trafficking. This endosomal protein regulates the cell-surface expression of epidermal growth factor receptor. This protein also has a role in sorting protease-activated receptor-1 from early endosomes to lysosomes.[22]
PIK3R3	B3-5p,B4-3p,B5-5p	ENSBTAG00000002979	Phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol and similar compounds, serving as second messengers in growth signaling pathways. PI3K is composed of a catalytic and a regulatory subunit. The protein encoded by this gene represents a regulatory subunit of PI3K. The encoded protein contains two SH2 domains through which it binds activated protein tyrosine kinases to regulate their activity.[6]
IGF1	B1-3p,B3-5p,B5-5p	ENSBTAG00000011082	Insulin_like growth factors; specific to vertebrates. Members include some peptides, including insulin-like growth factors I and II, which play a variety of roles in controlling processes such as growth, differentiation, and reproduction.[23]
NFAT5	B1-3p,B2-5p,B4-3p	ENSBTAG00000013412	Nuclear factor of activated T cells 5 The product of this gene is a member of the nuclear factors of activation. T cells family of transcription factors. Proteins belonging to this family play a central role in inducible gene transcription during the immune response. This protein regulates gene expression induced by osmotic stress in mammalian cells. Unlike monomeric members of this protein family, this protein exists as a homodimer and forms stable dimers with DNA elements.[24]
HIF3A	B1-5p,B2-3p,B4-3p	ENSBTAG00000018948	The protein encoded by this gene is the alpha-3 subunit of one of several alpha/beta subunit heterodimeric transcription factors that regulate many adaptive responses to low oxygen tension (hypoxia). The alpha-3 subunit lacks the transactivation domain found in factors containing either the alpha 1 or alpha 2 subunits. It is thought that factors containing the alpha 3 subunit are negative regulators of hypoxia-inducible gene expression.[25]

After total RNA extraction from the BLV+ samples and polyadenylation of the RNA samples, cDNAs were synthesized. We designed the primers (oligo7 and Allele ID-6 software) for miR-B4-3p and miR-B2-5p ourselves, however, they were manufactured by Bonyakhteh Co. Finally, qPCR was performed on the microRNA samples. Melting curve analysis was used to evaluate the specificity of the primers. According to the melting curve analysis, all the primers we designed were highly specific, and they had the same melting point at 80.00 ˚C. Amplification curve analysis was done to evaluate the miRNA samples cycle threshold (CT). The CT value for PL samples was lower than that of the AL samples and also the CT value of the lymphosarcoma samples was lower than the CT of samples from BLV+ with normal lymph nodes. This indicated that the miRNAs were highly expressed in the diseases BLV+ cattle with PL and lymphosarcoma form (Fig. 1). Expression levels of miR-B4-3p and miR-B2-5p in B lymphocytes of BLV+ cattle to PL was compared to AL (first group). In the present study, we found that the expression levels of both miRNAs were significantly higher in the PL cattle compared to AL cattle ($p < 0.05$). The FC value of miR-B4-3p expression in PL cattle compared to AL cattle was 22, whereas, it was 67 for miR-B2-5p expression. This indicated increased expression of these miRNAs in the PL cattle (Fig. 2) Expression levels of miR-B4-3p and miR-B2-5p in B lymphocytes of BLV+ cattle with lymphosarcoma was compared to BLV+ cattle with normal lymph nodes (second group): We observed a significantly increased expression level of both miRNAs in the lymphosarcoma cattle compared to the cattle with normal lymph nodes ($p < 0.05$). The FC value of miR-B4-3p expression in the lymphosarcoma cattle was compared to cattle with normal lymph nodes was 47, whereas, it was 133 for miR-B2-5p expression. This indicated increased expression of these miRNAs in the cattle with lymphosarcoma (Fig. 2).

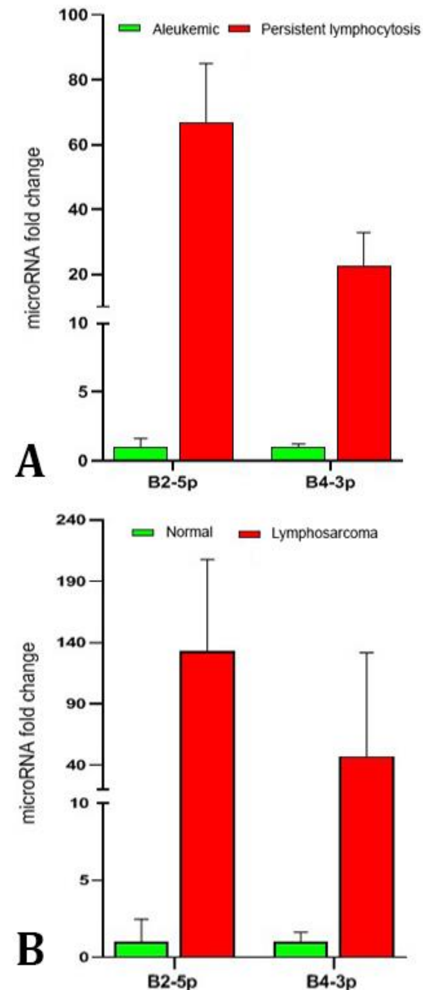

Fig. 2. A) Expression levels of two miR-B4-3p and B2-5p in PL animals was compared to AL animals. **B)** Lymphosarcoma was compared to cattle with normal lymph nodes. The expression of both miRNAs in the patient group (PL and lymphosarcoma) was significantly increased compared to AL and normal ($p < 0.05$).

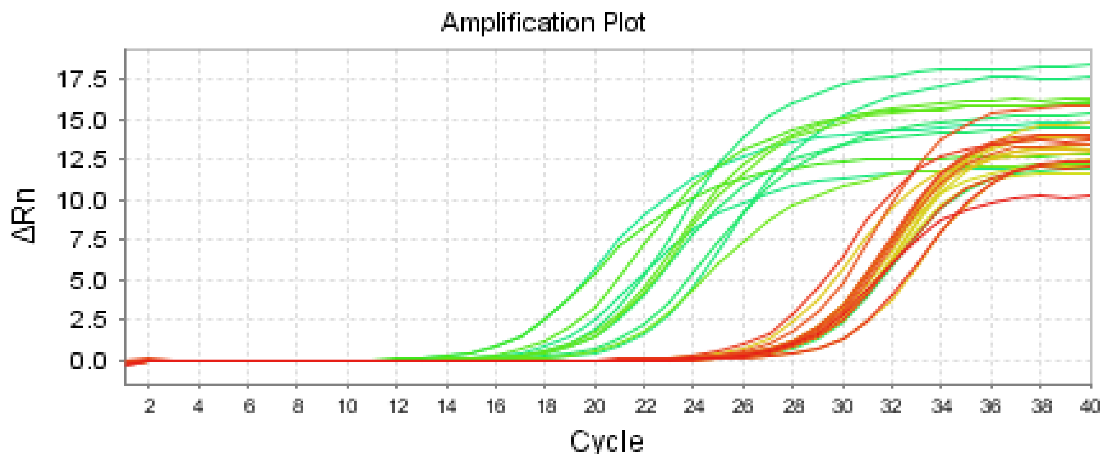

Fig. 1. The amplification plot of miR-B4-3p. The green curves with low cycling threshold (CT; 20-16) correspond to the specimens collected from the cattle with lymphosarcoma and the red curves with high CTs (30-26) correspond to the normal lymphoid tissue specimens.

Discussion

It is known that miRNAs play a major role in regulating several genes in the cell.[26] The expression of miRNAs in the bovine leukemic virus has been confirmed over the past years and this strengthens the hypothesis that miRNAs play an important role in the tumorigenesis and pathogenesis of this virus. Another important factor that adds to the importance of the BLV miRNAs is the difference in transcription enzymes between these miRNAs and other viral and cellular miRNAs. The BLV miRNAs are transcribed by RNA polymerase III, whereas most cellular and viral miRNAs are transcribed by RNA polymerase II. Because of this, the process of maturation of the BLV miRNAs is Drosha-independent[16] which may play a vital role in the pathogenesis of the virus. When the virus enters a B lymphocyte, it quickly translates its structural proteins and produces viral particles. However, after immune system activation and the production of anti-gp51 antibodies, the expression of viral proteins and synthesis of viral particles stop with time. However, studies have shown that the process of tumorigenesis of the virus continues even after immune system activation and due to the small size of the miRNAs, the immune system often does not detect them, therefore, they can be expressed in a large amount in the infected cell.[18] Our study results confirmed that the expression of the viral miRNAs was increased during BLV infection. We observed that the BLV miRNAs were highly expressed in PL and lymphosarcoma stages of BLV infections. As described earlier, even when the expression of the viral structural proteins, transcribed by RNA polymerase II stops, the expression of the miRNAs continues.[10] It seems that similar to the herpes virus miRNAs which downregulate the expression of herpesvirus structural proteins, the miRNAs of BLV also play an important role in downregulating the expression of viral structural proteins.[18] In addition to the inhibition of the expression of some cellular proteins, the BLV miRNAs may also influence the regulation of viral protein expression. However, little is known on the role of the BLV miRNAs in regulating viral and cellular protein expression, and further research is required. Researchers first began investigating the BLV miRNAs in 2012, and since then, these miRNAs have gained the attention of many investigators.[15,27] The sequences of the miRNAs of BLV were determined and have been registered in miRNA databases. The number of transcripts of each miRNA in cell cultures of infected cells was determined by deep sequencing.[16]

Three different miRNAs target several eight genes. Our studies showed that most of these genes play a role in suppressing tumors and pathways in intracellular metabolism. Therefore, if inhibited by viral miRNAs, they lead to the development of tumors or abnormal cell metabolism in the cell. Here is a brief description of these eight genes. For example, sorting nexin-1 (SNX1), the first member of the sorting nexins (SNXs) found in mammals, in which SNX1 could interact with the epidermal growth factor receptor (EGFR).[22]

Further works showed that SNX1 played an important role in cell endocytosis, efflux, protein sorting, cell signal transduction, membrane transport and remodeling, and organelle movement. It relates SNX1 to many intracellular signaling pathways. Down-regulation of SNX1 promotes HGF-induced MET (product of oncogene c-met) endocytosis leads to phosphorylation, and then activates the RTK/RAS signaling pathway resulting in cell proliferation activation inhibition of cell apoptosis.

Also, SNX1 is reported to be associated with the development and metastasis of some tumors, including lung cancer and colon cancer.[23] Nuclear factor of activated T cells 5 (NFAT5) is a member of the Rel family of transcription factors (TFs) that shares a conserved DNA-binding domain with NFATc1-4 and NF-kB. Therefore, NFAT5 has important roles in different tissues normally exposed to hypertonicity such as kidneys, skin and eyes. It has also been implicated in several physiologic and pathologic conditions including cancer cell proliferation and invasion.[24-28] The insulin-like growth factor-I (IGF-I), somatomedin C, is cellular and secreted growth factor critical for normal body growth, development and maintenance and has important roles in multiple biological systems. A variety of cellular responses are induced by IGF-I including cell proliferation, differentiation, migration and survival. These cellular responses have implicated IGF-I in several conditions such as the pathophysiology of several cancers, and unlike other growth factors, IGF-I act as both a mitogen and a differentiation factor.[23,29] The PIK3R3 (phospho-inositide 3 kinase regulatory subunit 3), a member of the phosphatidylinositol 3-kinase (PI3K) family, has been suggested to play crucial roles in diverse biological processes, such as cell proliferation, differentiation, carcinogenesis and tumor angiogenesis.[30] The SMARCD1, a member of the SWI/SNF chromatin remodeling complex family, regulates gene transcription by binding specific transcriptional factors and altering local chromatin structure.[21]

The MLL2 gene encodes histone methyltransferase and also interacts with RNA polymerase II. 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.[20] The TET3 gene (ten-eleven translocations) is a member of the deoxygenase family. The enzyme converts 5-methyl cytosine (5mc) to 5-hydroxymethyl-cytosine (5hmc).[31] Also, the family of hypoxia-inducible factors, including HIF-3α, are believed to play key roles in angiogenesis and metabolism variation in DNA methylation of the associated CpG sites has been linked

with altered gene expression.[25] Therefore, changes in the expression of these genes can lead to the disruption of cellular processes. Therefore, it is necessary to investigate the relationship between the expression of these genes with BLV-miRNA *in vivo* conditions.

Among them, 760 genes (50.00%) were targeted by miR-B4-3p. This finding was consistent with that of previous studies. In a previous study, B4-3p had the highest copy number in BLV-infected B lymphocytes accounting for 62.00% of the BLV-miRNA transcripts in the infected cells. The copy number of miR-B4-3p in infected cells is so high that it accounts for the highest copy number among the total miRNAs in infected B lymphocytes and even more than miR-17-92- a cellular miRNA highly expressed in B-cell lymphomas.[32] The miR-B4-3p is arguably the most important BLV micro RNA because of its high number of target genes and high copy number (transcripts) in infected B lymphocytes as well as the significant increase in its expression in cattle infected with PL and lymphosarcoma compared to asymptomatic and normal cattle. Therefore, conducting a study on the B4-3p gene targets seems to be more important than other BLV miRNAs. We observed that most of the target genes identified for these miRNAs often played important roles in intracellular signaling—for example, TP53, PTEN, CDK6. FOX and TET3 were tumor suppressor genes in the cell, and when their expression was decreased, tumor progression was occurred that was consistent with the tumorigenesis of the BLV. However, because of the abundance and diversity of the target genes of each BLV-miRNA, many positive and negative roles can be noted for them. For example, miR-B4-3p has the same target genes as miR-29, because of the similarity of the "seed" region. In recent years miR-29 has emerged as a critical miRNA in various cancers, and it has been shown to regulate multiple oncogenic processes including epigenetics, metabolism, proliferation, apoptosis, metastasis, fibrosis, angiogenesis and immunomodulation.[33]

Although miR-29 has been thoroughly documented as a tumor suppressor in most studies, some controversy remains with conflicting reports of miR-29 as an oncogene. Some studies indicated an important role in the healthy and sick individual immune system regulation. Studies have shown that miR-29b controls innate and adaptive immune responses by targeting IFN-γ produced mRNA.[34] Therefore, there is a hypothesis that BLV-infected cattle that produce high levels of miR-B4-3p have a weak immune response because of the disruption of intracellular messenger pathways and inhibition of genes involved in immune responses such as NFATC4, IL-17RD, IGF1, CD276. Because of the complexity of intracellular processes and the direct and indirect effects of each of these miRNAs on intracellular pathways, it is the results should be confirmed in *in vivo* conditions. In one study in 2018, it was found that the expression level of IgM antibodies in BLV+ cattle was significantly decreased compared to BLV- cattle due to the targeting of genes involved in the immune response. The researchers further explained that targeting PAX5, BCL6, BLMP1 and IGj genes resulted in a decrease in their expression and subsequently a decreased production of IgM.[18] In another study in 2016, the expression levels of several genes involved in apoptosis and cellular immunity such as FOS, GZMA, PPT1 in cell cultures of virus-infected cells were compared to control samples. It was found that the expression of these genes was decreased in the infected cells.[35]

Another miRNA we considered for further evaluation in our study was the BLV-miR-B2-5. This miRNA was the second most expressed BLV-miRNA after miR-B4-3p in infected B lymphocytes. MiR-B2-5p accounted for 15.00% of the total BLV miRNAs in infected B lymphocytes in a previous study.[16] Another important point about miR-B2-5p is the similarity of its seed region to the seed region in miR-943 in humans. In humans, the expression of miR-943 is inversely associated with the expression of the P53 tumor suppressor gene. Thus, blocking or inhibition of p53 expression leads to an increased level of expression of miR-943. In the present study, we found a significantly increased miR-B2-5p expression in the BLV+ cattle with PL and lymphosarcoma compared to BLV+ cattle that were asymptomatic and with normal lymph nodes. Also, sequence analysis in our study showed that miR-B2-5p targeted 102 genes, and given its importance, it is necessary to investigate its effects on its target genes.

Viruses are estimated to cause 15.00 - 20.00% of all human cancers. The infective agents most frequently investigated in connection with human breast cancer are human papillomavirus (HPV), Epstein Barr virus (EBV), and murine mammary virus (MMTV).[17] Recently, bovine leukemia virus (BLV) has been added to the virus candidates. The way BLV could enter the cells and infect them remains unknown. A recently-published paper showed the interaction of bovine AP3D1, the putative cell surface receptor and the viral 51.00 kDa glycoprotein (gp51).[27] This model suggested receptor-ligand interactions, which could occur in BLV infection and lead to viral binding and fusion regarding viral entry. The BLAST analysis of boAP3D1 predicted 88.00% identity with human protein for AP3D.[17] How humans become infected with BLV is not known.

Transmission from cattle to humans is plausible, as BLV is widespread in beef herds and dairy herds. Although pasteurization renders the virus non-infectious, many people have drunk raw milk and/or eaten raw beef in their life. Breast cancer incidence is markedly higher in countries with high milk consumption.[8,36] One potential challenge confronting the elucidation of BLVs transmission route to humans is the long agricultural association of humans with cattle, which began over 2,000 years ago,

while milk pasteurization in western countries was not standard practice until around 1925. The virus may have been transmitted to humans in this way and integrated into the human genome. Therefore, the current reservoir for transmission to humans could be cattle and humans.[8] According to previous studies, fragments of the BLV virus genome such as gag, env, tax genes have been identified in breast cancer tissue samples. Because other than the tax gene, other genes do not have tumorigenic properties. Therefore, it is hypothesized that another factor must be involved in tumorigenesis in which miRNAs may be involved in the development of breast cancer. Although no studies have been performed in this field, it needs more research.

The present study highlighted the importance of the BLV miRNAs. Our study results indicated 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. Also, we found that the expression levels of miR-B4-3p and miR-B2-5p were significantly increased in BLV+ cattle with PL form compared to the Al form of the disease ($p < 0.05$). Also, the expression levels of these miRNAs were significantly increased in the BLV+ cattle with lymphosarcoma compared to BLV+ cattle with normal lymph nodes, indicating that despite the lack of expression of virus structural proteins and the termination of viral particle production, miRNAs were 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 hypothesized that the miRNAs of BLV influenced the regulation of tumor suppressor genes in the cell, and the inhibition or decreased expression of these genes was associated with the tumorigenesis of the virus. The authors of this paper also emphasized that given the confirmation of the expression of BLV genes in breast cancer, it was necessary to investigate the expression of the BLV miRNAs in breast cancer patients for a possible association. In veterinary medicine, studies are still needed to investigate the association of the BLV miRNAs with the expression of their target genes in infected animals.

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Conflict of interest

The authors declare no conflict of interest.

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