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Bovine Leukaemia Virus Tax Antigen Identification in Human Lymphoma Tissue: Possibility of Onco-protein Gene Transmission

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ABSTRACT

Background: Bovine Leukaemia Virus (BLV) is the cause of Enzootic Bovine Leukosis (EBL) which belongs to retroviruses including Human T-cell leukaemia virus and simian T-lymphotropic virus. Due to this familiarity, the possibility of BLV transfer from animal production to humans may exist.

Materials and Methods: In the present study, formalin-fixed, paraffin-embedded and fresh human lymphomas tissues were used for detecting the BLV Tax genome and BLV Tax expression using nested-PCR and RT-nested-PCR.

Results: Nested-PCR evaluation showed that 9 of 41 samples contained Tax region of BLV genome, of which eight samples belonged to high grade diffuse large cell lymphoma (B-cell type). Also, the results of RT-nested-PCR showed the BLV Tax expression in 2 of 5 high grade diffuse large cell lymphoma (B-cell type) samples.

Conclusion: These findings explain the possible relation between BLV infection and the occurrence of some types of human lymphomas for the first time.

Introduction

Bovine Leukaemia Virus (BLV) belongs to the *Retroviridae* family and it is an agent for Enzootic Bovine Leukosis (EBL) [1]. Bovine leukosis was first reported by Leisering (1871), who described the presence of non-suppurative nodules in enlarged spleen [2]. This virus belongs to the oncogenic retroviruses including human T-cell leukaemia virus types 1, 2, and 3 (HTLV-1, HTLV-2, and HTLV-3), and Simian T-lymphotropic Virus (STLV) [3]. These groups of retroviruses encode Tax protein, which transformed the infected cells [4].

In addition to gag, pol and env genes which are required for the synthesis of viral particle, the BLV genome contains X region, located between the envelope gene and the 3′ long terminal repeat (3′-LTR). This region contains some genes

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including Tax, Rex, R3, and G4 [1] that the Tax and Rex genes are involved in regulation of viral transcription [5].

The lymphoid neoplasms in human encompass a group of entities that vary widely in terms of their clinical presentation and behaviour. Others, the lymphomas, typically appear as tumour masses within either lymph nodes or other organs. Two groups of lymphomas are recognized: Non-Hodgkin's Lymphomas (NHLs) and Hodgkin lymphoma that both arise in the lymphoid tissue [6]. Unfortunately, the aetiology of most cases of NHLs is unknown, although several genetic, environmental and infectious agents have been associated with the development of these types of lymphomas [7, 8].

Milk of infected cows commonly contains the viral particles, which is the root of transmission for chimpanzees [9]. Recently, a study on 96 Australian women by Buehring et al. showed the presence of virus DNA in 41 and 80% of healthy and breast cancer women, respectively [10]. Whereas, the risk of developing leukaemia for workers of livestock farm and meat processing is controversial [9].

In the current study, we have hypothesized that BLV can be one of the infectious causes of some types of human lymphomas and the main objective of this study was, therefore to investigate and monitor the BLV Tax genome in some types of human lymphoid malignancies.

Materials and Methods

The BLV genome in viral particle is composed of RNA that changes to DNA when the virus integrates to the host cell genome as proviral DNA. Thus, proviral DNA extraction procedures were performed on paraffin-embedded tissues. Special precautions were taken to avoid contamination and all steps were carried out in separate rooms, all pipetting procedures were performed under Plexiglas boxes and rooms were pre-irradiated with ultra violet light [11].

Tissue samples

The present study was conducted on forty-one formalin-fixed, paraffin-embedded and 5 fresh human lymphoma tissues (Laboratory of Pathology, Iran Mehr Hospital and Khatamolanbia Hospital, Iran). By using sterile blades, two slices were prepared and collected in the labelled sterile microtubes. Samples of fresh tissues were also prepared and kept in liquid nitrogen.

RNA extraction from paraffin-embedded tissues

Samples of Formalin-Fixed Paraffin-Embedded (FFPE) tissues in labelled microtubes were deparaffinized by washed for 30 min in 1 ml xylene and twice for 30 min with 15 min intervals in 100% ethanol and 70% ethanol, respectively at room temperature. After air drying, each of the sections was incubated with 500 µl of White Cell Lysis Buffer (Wclb) (100 mM Tris-HCl [pH 7.6], 40 mM EDTA [pH 8.0], 50 mM NaCl, and 0.2% sodium dodecyl sulphate) and 3 µl proteinase K at 55°C for 2 h. In the next step, 200 µl saturated-6M NaCl was added to each microtube and all the samples were cooled for 15 min in -20°C. The samples were centrifuged for 15 min in 12000 g at 4°C, the upper aqueous phase was transferred to a new tube, mixed with 1 volume of isopropanol. After centrifugation for 15 min in 12000 g at 4°C, the upper aqueous phase was thrown away and DNA was ethanol precipitated. DNA pellet was dissolved in 30 ml distilled water and the solution was stored at -20°C for nested-PCR reaction [12].

RNA extraction from fresh tissues

For total RNA extraction from fresh samples the RNX-plus solution were used (Cinnagen, Iran, Cat. No. RN7713C). One millilitre of RNX-plus solution was added to pre-homogenized sample and incubated at room temperature for 5 min. After that, 200 µL chloroform was added and centrifuged in 12,000 rpm at 4°C for 15 min. The upper phase was separated, equal volume of cold isopropanol added to it and centrifuged in 12,000 rpm for 15 min. Resulting pellet was then washed via using 70% ethanol. The extracted RNA was dissolved in Diethyl Pyrocarbonate (DEPC)-treated water and stored at 70°C.

cDNA synthesis

By using reverse transcription method, cDNA was synthesized. A mixture containing total RNA (10 µL), primers (0.5 µM final concentration), and sterile DEPC solution (1µL) were incubated at 65°C for 5 min. The mixture was then briefly quenched on ice and mixed with additional mixture containing 1µL of M-MLV reverse transcriptase (BIONEER, South Korea, Cat. No. E-3121-CFG), 2 µL dNTP and 4 µL RT reaction buffer. The RT reaction was carried out at 42°C for 1 h and terminated by incubation at 70°C for 10 min. Then microtubes were stored at -20°C for nested-PCR reaction.

Table 1. Primers used for PCR and nested PCR amplification of BLV proviral genome

Primers Type	Primer	Sequences (5'-3')
(First round PCR)	Forward (Txf)	ATGCCTGGTGCCCCCTCT
	Reverse (Txr)	ATTGGCATTGGTAGGGCTGG
(Nested PCR)	Forward (FwinTX)	GAAAGGATCGACACCACGCTC
	Reverse (RewinTX)	ATTGGCATTGGTAGGGCTGG

Primers design

The primers for both nested-PCR and RT-nested-PCR were designed according to sequence reference number EF600696 on NCBI GenBank (Table 1).

Nested-PCR assay

Polymerase chain reaction was performed using 3 µL of cDNA with 1 Unit per reaction of Taq DNA polymerases (Cinnagen Co. Cat. No. TA8109C) (500 U/µL), 50 mM of MgCl2, 2.5 µL of PCR buffer (10X), 0.5 µL of dNTP, 0.5 µL of each primer (20 pmol/L), and 18.1 µL of sterile double distilled water.

PCR program was planned using thermocycler (Techne TC-512) as follows: a heated lid (105°C for 4 min) and after that the reaction mixtures were subjected to 40 cycles of amplification by using the following program: (94°C for 30 s, 58°C for 30 s, and 72°C for 30 s); This was followed by a final extension step at 72°C for 5 min. For nested PCR, 0.5 µl of the first PCR product was amplified using the same reaction conditions that were described previously.

Analysis of amplified cDNA

A total of 5 µl of each PCR product was mixed with 1 µl 6X loading buffer (Fermantes Co. Cat. No. #R0611) and was subjected to 8% acrylamide gel electrophoresis in 1X TBE buffer (10.8 gr Tris-acetate, 4 ml EDTA, 5.5 gr Boric acid dissolved in 1000 ml distilled water) for 80 min at 90V. Silver staining protocol was carried out for detection of PCR products in gel. In order to confirm the PCR conditions, 20 µL of PCR products was applied for sequencing by Applied Biosystems 3100 ABI (Macrogen Co. Korea).

Analytical procedure

Data were analysed using SigmaStat V. 3.1 program and the descriptive statistics and percentages were reported.

Results

Outer and inner primers (Txf,Txr and FwinTX, RewinTX) were used for both generated products of nested-PCR and RT-nested-PCR with the calculated size of a 220 bp (Figure 1 and Figure 2). The mentioned size was estimated according to the size of positive control (pure cDNA of cultured virus). No products were generated with negative control.

Among forty-one human lymphoid paraffinized tissue specimens, 9 of them and among 5 human lymphoid fresh tissue specimens, 2 of them were positive for BLV

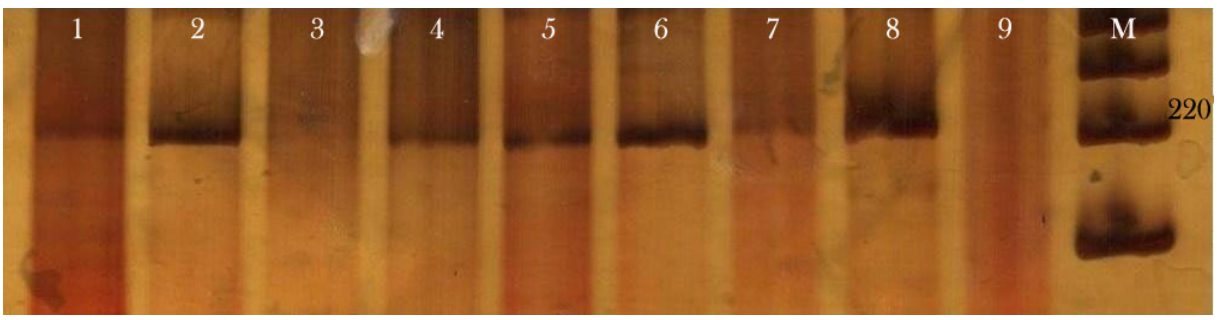

Figure 1. Nested PCR results (Acrylamide gel, Silver staining)
Lane M, molecular mass marker; lane 9 and 8 represent negative control (no template cDNA) and positive control, respectively; lanes 1, 2, 4, 5, 6, and 7 positive samples; lane 3 negative sample

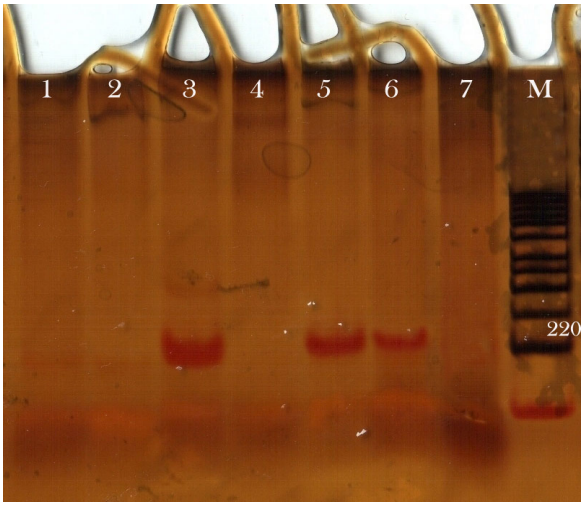

Figure 2. RT-nested PCR results (Acrylamide gel, Silver staining)

Lane M, molecular mass marker; lane 7 and lane 6 represent negative control (no template cDNA) and positive control, respectively; lanes 3 and 5 positive samples; lanes 1, 2, and 4 negative samples

proviral DNA using nested PCR and RT-nested-PCR, respectively (Table 2 and Table 3). These lymphoid tissues were obtained from lymph nodes of different parts of the body. Pathological diagnosis of these infected tissues had shown different types of lymphoid neoplasms. Among the presented types of lymphomas and from 9 positive paraffinized samples, 8 blocks with the relative frequency of 19.51%, and 1 block with the relative frequency of 2.44% belonged to high grade diffuse large B-cell lymphoma and Burkitt's lymphoma, respectively. Also, from 5 fresh samples, 2 with relative frequency of 40% belonged to high grade diffuse large B-cell lymphoma.

Discussion

It has been estimated that twenty percent of human cancer is related to viral infection. Viral oncogenic abilities are related firstly to the generation of genomic instability and secondly to the increase in the rate of cell proliferation. Viruses also resist to apoptosis and alter the DNA repair mechanisms which finally cause the cell polarity changes and often alter the antiviral immune response [8]. Viruses may induce cancer via causing immunosuppression, chronic inflammation and chronic antigenic stimulation.

Furthermore, International Agency for Research on Cancer (IARC) classified the Epstein Barr Virus (EBV), Kaposi's Sarcoma-associated Herpesvirus (KSHV), Human High risk Papillomaviruses (HPV), Merkel Cell Polyomavirus (MCPV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human T-cell Lymphotropic Virus type 1 (HTLV1) as type 1 carcinogenic agents. 8 In this regard, it is possible that other viruses like BLV may have a role in human cancers. For the past three decades, BLV has not been regarded as a threat to humans, until Lawson et al. announced that bovine leukaemia virus gene sequences have been found by Giovanna et al. Buehring et al. and Buehring et al. in human breast cancer [10, 13-16].

Table 2. Positive specimens for BLV proviral DNA in paraffin-embedded human samples with lymphoma

Sample No.	Histopathological Diagnosis	Sampling Location	Age/Sex	Nested-PCR Results
1	Low grade diffuse small cell lymphoma, B-cell type	neck	60/F	_
2	Peripheral T-cell lymphoma	neck	72/M	_
3	Mantle cell lymphoma	post auricular	54/M	_
4	Follicular lymphoma	neck	37/F	_
5	Low grade diffuse small cell lymphoma, B-cell type	pelvis	23/F	_
6	Low grade diffuse small cell lymphoma, B-cell type	neck	68/F	_
7	Peripheral T-cell lymphoma	bone marrow	45/F	_
8	Lymphoblastic lymphoma, T-cell type	neck	31/M	_
9	Large cell anaplastic lymphoma, T-cell type, high grade	neck	39/M	_
10	Mantle cell lymphoma	neck	76/M	_

Sample No.	Histopathological Diagnosis	Sampling Location	Age/Sex	Nested-PCR Results
11	Burkitt's lymphoma	inguinal canal	43/F	+
12	High grade diffuse large cell lymphoma, B-cell type	Gastro-intestine	40/F	_
13	Low grade diffuse small cell lymphoma, B-cell type	bone marrow	45/M	_
14	High grade diffuse large cell lymphoma, B-cell type	mediastinum	16/M	_
15	High grade diffuse large cell lymphoma, B-cell type	neck	71/F	_
16	Peripheral T-cell lymphoma	neck	35/M	_
17	low grade small cell lymphoma, B-cell type	axillary lymph node	72/M	_
18	High grade diffuse large cell lymphoma, morphologically B-cell type	tonsil	66/F	_
19	High grade diffuse large cell lymphoma, B-cell type	inguinal canal	52/F	+
20	High grade diffuse large cell lymphoma, B-cell type	axillary lymph node	84/M	+
21	Lymphoblastic lymphoma, T-cell type	neck	24/M	_
22	High grade diffuse large cell lymphoma, B-cell type	gastrointestine	71/F	+
23	High grade diffuse large cell lymphoma, B-cell type	tonsil	60/M	+
24	High grade diffuse large cell lymphoma, B-cell type	neck	75/F	+
25	High grade malignant lymphoma, B-cell type	neck	71/M	+
26	High grade diffuse large cell lymphoma, B-cell type	tonsil	76/M	+
27	High grade malignant lymphoma, diffuse large B cell type	supraclavicular lymph node	69/M	+
28	Small round cell tumor consistent with low grade B-cell lymphoma	mediastin	67/M	_
29	low grade small cell lymphoma, B-cell type	inguinal canal	63/M	_
30	Lymphoblastic lymphoma, T-cell type	Large intestine	43/M	_
31	low grade diffuse small cell lymphoma, B-cell type	Neck	69/F	_
32	low grade diffuse small cell lymphoma, B-cell type	Neck	59/F	_
33	Mantle cell lymphoma	spleen	76/F	_
34	Large B-cell lymphoma, lymphomatoid granulomatosis	kidney	27/F	_
35	Lymphoblastic lymphoma, T-cell type	neck	29/F	_
36	low grade diffuse small cell lymphoma, B-cell type	_	39/F	_
37	low grade small cell lymphoma, B-cell type	neck	33/M	_
38	Peripheral T-cell lymphoma	neck	52/M	_
39	Lymphoblastic lymphoma, T-cell type	bone marrow	23/M	_
40	Diffuse small lymphocytic lymphoma, morphologically B-cell type, low grade	axillary lymph node	78/F	_
41	Anaplastic large cell lymphoma, high grade, T-cell type	axillary lymph node	28/M	_

F: Female; M: Male

Table 3. Positive specimens for BLV proviral DNA in fresh human samples with lymphoma

Sample No.	Histopathological Diagnosis	Sampling Location	Age/Sex	RT-nested-PCR Results
1	High grade malignant lymphoma, diffuse large B cell type	Supraclavicular lymph node	69/M	+
2	Peripheral T-cell lymphoma	Bone marrow	_	_
3	Lymphoblastic lymphoma, T-cell type	Neck	50/F	_
4	Low-grade B-cell lymphoma	Mediastinum	67/M	_
5	Non-Hodgkin's lymphoma, Diffuse large B-cell type; High grade	Neck	75/F	+

F: Female; M: Male

A human serological survey by using immunoblot test, showed the presence of BLV antibody in 74% of samples, which could be a response to BLV antigens in heat-treated milk or meat and direct contact with cattle [9]. This indicates that people are exposed to antigens derived from BLV. The potential human health hazards associated with BLV have been a constant concern of the meat and dairy industries [17, 18]. Cohort and case-control studies suggest that higher intake of meats and dietary fat increased the risk of NHLs [19]. The fifth most common systemic human cancer is Lymphoma, in which Diffuse Large B-cell Lymphoma (DLBL) is the most common subtype [6].

Our study was performed on paraffinized and fresh lymphoid tissues of NHLs patients using nested-PCR and RT-nested PCR for detection of BLV Tax genome. Results showed a relative frequency of 21.95% and 40% BLV genome in paraffinized and fresh samples, respectively. Previously, Mannetje et al. showed an elevated non-Hodgkin's lymphoma (NHLs) risk among meat processors but none of these studies has shown any direct correlation between the observed malignancies and BLV infections in the cattle present on the farm [20]. Recently, some researchers established a linked between the presence of BLV genome in humans and breast cancer but the current study went a bit further and for the first time confirmed the presence of Tax genome in some types of human lymphomas [10, 21]. The presence of BLV Tax genome was confirmed by nested-PCR assay in mostly high-grade diffuse B-cell type lymphoma (8 of 41 samples), Burkitt's lymphoma (1 of 41 samples) and one type of lymphoma in RT-nested-PCR assay. High-grade diffuse B-cell type lymphoma (2 of 5 fresh samples) was also detected. Furthermore, the BLV Tax gene was confirmed by sequencing the nested-PCR products of positive samples.

Tax region of BLV disrupts the regulation of cellular gene expression and alters cellular processing, DNA replication, DNA repair and apoptosis, leading to immortalization, transformation and leukemogenesis [22]. Merimi et al. suggested that silencing is critical for tumour progression and identified two distinct mechanisms involved in the complete suppression of virus and Tax expression in the BLV-injected sheep [23]. Usui et al. also showed that vaccination with BLV Tax-coding DNA induced protective effect against BLV in the early phase of infection [24].

These findings show the BLV's direct involvement in formation of some types of NHLs for the first time. The results of present study along with other studies indicate the possibility of BLV transmission to humans and as a result the necessity for veterinary authorities to encourage BLV eradication programs in dairy industry.

Conclusion

The research proved that the Tax region genome of Bovine leukemia virus exists in two types of human lymphomas (DLBL and BLs). So, BLV is associated with human lymphoma and bovine leukosis should be eradicated from dairy farms.

Ethical considerations

Compliance with ethical guidelines

All ethical principles were considered in the study.

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Authors contribution

Data collection, PCR analysis, and writing: All authors; Design, analysis: Gholam Ali Kojouri; PCR performing: Gholam Ali Kojouri; Revisision: Abdolnabi Kojouri.

Conflict of interest

The authors declared no conflict of interest.

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