NOTE *Internal Medicine*

Detection of monoclonal integration of bovine leukemia virus proviral DNA as a malignant marker in two enzootic bovine leukosis cases with difficult clinical diagnosis

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ABSTRACT. Monoclonal integration of bovine leukemia virus (BLV) proviral DNA into bovine genomes was detected in peripheral blood from two clinical cases of enzootic bovine leukosis (EBL) without enlargement of superficial lymph nodes. A BLV-specific probe hybridized with 1 to 3 *EcoRI* and *HindIII* fragments in these 2 atypical EBL cattle by Southern blotting and hybridization, as well as in 3 typical EBL cattle. The probe also hybridized to a large number of *EcoRI* and *HindIII* fragments in 5 cattle with persistent leukosis. These results suggest that the detection of monoclonal integration of BLV provirus into the host genome may serve as a marker of monoclonal proliferation and malignancy in difficult to diagnose EBL cattle.

KEY WORDS: atypical enzootic bovine leukosis, diagnostic marker, monoclonal integration

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Enzootic bovine leukosis (EBL) is a malignant hematopoietic disease of cattle caused by bovine leukemia virus (BLV) [1, 7, 12]. Clinical signs of EBL are non-specific and depend on the affected organ. Anorexia, depression, decreased productivity, enlarged superficial lymph nodes and lymphocytosis are common findings in EBL [1, 7, 13]. Definitive diagnosis of EBL requires the demonstration of malignant cells in enlarged superficial lymph nodes or the presence of neoplastic lymphocytes in peripheral blood with lymphocytosis [7]. Fine needle aspiration (FNA) of enlarged lymph nodes is usually performed to diagnose EBL, but the sensitivity and specificity of FNA are not always reliable with 38 to 67% and 25 to 80%, respectively [18]. Another study reported that abnormal lymphocytes (lymphoblast) were identified in only 10.4% of clinical cases of bovine leukosis [3]. It is often difficult to diagnose the onset of EBL in cases which do not present with lymphocytosis or enlarged lymph nodes [7, 14]. In such cases, definitive diagnosis would be undetermined until necropsy. Recently, more reliable markers for diagnosing EBL onset have been reported. For example, serum thymidine kinase (TK) activity is a potential biomarker for the clinical diagnosis of EBL, but requires a radioimmunoassay test [13, 17]. Expression of certain genes, such as interleukin 2 receptor and Wilms' tumor genes, in peripheral blood may also be candidate clinical markers for malignancy. However, these are associated with low sensitivity and specificity [16]. Thus, other methods are required for early diagnosis of the onset of EBL.

Human T-cell leukemia virus-1 (HTLV-1) is closely related to BLV and causes adult T-cell leukemia (ATL). Monoclonal integration of HTLV-1 into tumor cells has been demonstrated in all types of ATL by Southern blotting [19, 21]. This finding suggests the monoclonal proliferation of lymphocytes in patients with ATL and is used as a marker of malignant transformation of infected lymphocytes [4, 15]. Similarly, integration of BLV has also been studied in cattle with EBL [6, 8–11]. Proviral DNA reportedly integrates at one or a few sites into the genomes of neoplastic cells in host cattle with lymphosarcoma [8, 11]. In contrast, proviral DNA integrates into a large number of genomic sites in cattle with persistent lymphocytosis, which is considered the pre-stage of EBL [8]. These findings suggest that the integration patterns of BLV provirus into bovine cells may serve as a diagnostic tool for the malignant transformation of host cells infected with BLV. Although typical clinical cases of EBL with obvious enlargement of superficial lymph nodes were used in these previous studies, the clinical utility of this diagnostic method for EBL cattle with no lymphadenopathy or lymphocytosis (atypical EBL) has never been evaluated. Thus, in the present study, we evaluated the utility of analyzing the pattern of BLV provirus integration as a marker for EBL onset in cattle without enlarged superficial lymph nodes.

Two atypical difficult to diagnose EBL cattle were used (Table 1). The first case (ATY-1) was a 7-year-old Holstein cow which presented with fever, cough, wheezing, difficult

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Table 1. Profiles of 2 atypical of enzootic bovine leukosis (EBL) cattle and 13 control cattle

Group and ID	Breed	Sex	Age (months)	White blood cell count (/μl)	Lymphocyte count (/μl)	BLV antibody	TK (U/l)	Clinical findings
Atypical EBL without lymphadenopathy								
ATY-1	HF	F	94	12,900	6,579	+	51.0	Fever, cough, wheezing, astasia
ATY-2	HF	F	72	13,400	9,916	+	52.5	Anorexia, astasia
Control Animals								
Typical EBL								
TY-1	HF	F	89	266,700	258,699	+	1000.0	Anorexia, depression, anemia, diarrhea, astasia
TY-2	HF	F	50	104,000	102,960	+	1000.0	Anorexia, anemia, lethargy, lymphadenopathy
TY-3	HF	F	21	46,200	43,890	+	40.0	Anorexia, depression, lymphadenopathy
Persistent lymphocytosis								
PL-1	HF	F	69	16,700	10,251	+	NT	Clinically healthy
PL-2	HF	F	54	14,400	9,792	+	NT	Clinically healthy
PL-3	HF	F	53	14,300	11,011	+	NT	Clinically healthy
PL-4	HF	F	46	14,700	11,319	+	NT	Clinically healthy
BLV infection without persistent lymphocytosis								
BLV-1	HF	F	66	8,300	5,395	+	NT	Clinically healthy
BLV-2	HF	F	64	7,500	4,050	+	NT	Clinically healthy
BLV-3	HF	F	61	6,900	3,657	+	NT	Clinically healthy
BLV-4	HF	F	53	9,700	6,208	+	NT	Clinically healthy
BLV-5	HF	F	41	8,600	4,730	+	NT	Clinically healthy
Clinically normal without BLV infection								
N-1	HF	F	51	8,600	5,676	–	NT	Clinically healthy

HF: Holstein Friesian, F: female, BLV: bovine leukemia virus, TK: thymidine kinase, NT: not tested.

breathing and astasia. No lymphadenopathy was noted by surface and rectal palpation. Increased white blood cell counts (12,900/μl) and slight lymphocytosis (6,579/μl) were found on blood examination, although atypical lymphocytes were not identified in a blood smear. Positive BLV infection was determined with an agar gel immunodiffusion (AGID) kit (Kitasato Institute Research Center for Biologicals, Kitamoto, Japan). Onset of EBL was suspected given a high serum TK activity of 51.0 U/l. However, the tentative diagnosis was laryngitis due to the lack of direct evidence of EBL. Necropsy findings included enlargement of mesenteric and abomasum lymph nodes, and a large mass in the larynx. The second case (ATY-2) was a 6-year-old Holstein cow which was examined by a local veterinarian. The cow presented with sudden anorexia and astasia. No lymphadenopathy was noted by surface and rectal palpation. Increased white blood cell counts (13,400/μl) and lymphocytosis (9,916/μl) were found on blood examination, but atypical lymphocytes were absent. Although the cow was BLV-positive by AGID and had high TK activity (52.5 U/l), EBL onset was not confirmed before necropsy. Necropsy findings included enlargement of several lymph nodes in the abdominal cavity. Microscopic examination led to a final diagnosis of EBL for these 2 cases.

A total of 13 cattle were also used as control animals (Table 1). Three cattle with typical EBL (TY-1 to −3) with obvious enlargement of superficial lymph nodes and/or remarkable lymphocytosis of more than 100,000/μl, were used as positive controls (Table 1). In all these typical cases, malignant cells were detected in both enlarged lymph nodes and peripheral blood, and EBL was confirmed by pathological findings and positive BLV infection. The other 10 clinically normal cattle were used as negative controls (Table 1), including 4 which were positive for BLV antibody and had persistent lymphocytosis (PL-1 to 4), 5 which were positive for BLV antibody but without persistent lymphocytosis (BLV-1 to 5) and 1 which was BLV negative and did not have persistent lymphocytosis (N1). Numbers of peripheral lymphocytes were evaluated by Bendixen's key criteria [2]

Genomic DNA of each cow was extracted from 200 μl of peripheral blood with the QIAamp® DNA Mini Kit (QIAGEN® GmbH, Germany) and kept at −30°C until analysis. Approximately 10 μg of genomic DNA from each cow was digested with *EcoRI* or *HindIII* (Roche Diagnostics, Mannheim, Germany) overnight. The digested DNA was run on 1.0% agarose gels at 50 V for 7.5 hr. After electrophoresis and washing the gel with distilled water, DNA fragments were transferred from agarose gels to a hydrophobic polyvinylidene difluoride (PVDF) membrane (RPN303B; Amersham Hybond™-P, GE Healthcare, Buckinghamshire, U.K.), as described in a previous report [6].

To prepare the BLV *tax*-specific probe for hybridization analysis, a 511 bp fragment of the BLV provirus *tax* gene was amplified from the peripheral blood of one BLV-positive cow by polymerase chain reaction (PCR) using the following primers: BLVtax-105F (5′-G AGC GAC TCC AAT TCG AAA G-3′) and BLVtax-616R (5′-GCT CGC CTA GGG GTA GAA TA-3′). PCR was performed as described previously [20] with an annealing temperature of 55°C using the Gene Amp® PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A.). The PCR product was purified with the

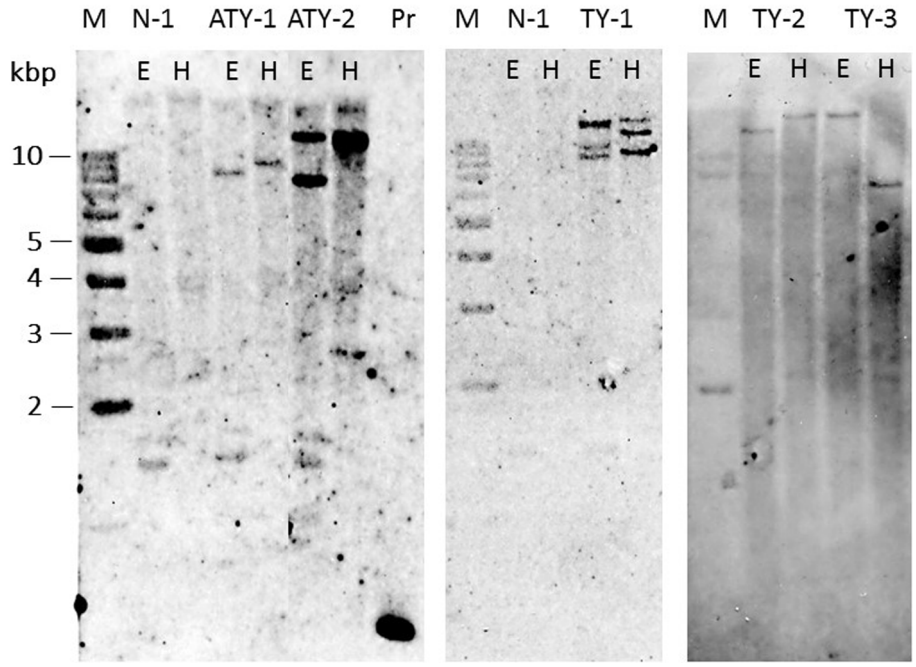

Fig. 1. Hybridization of the BLV *tax* probe to *EcoRI* and *HindIII* fragments in 2 atypical and 3 typical EBL cases. The probe hybridized with 1 to 3 *EcoRI* and *HindIII* fragments in these cases. ATY-1 and 2: Atypical EBL. TY-1, 2 and 3: Typical EBL cases with obvious lymphadenopathy and/or remarkable lymphocytosis. N-1: Negative controls without persistent lymphocytosis or BLV infection. H: *EcoRI* digestion, H. *HindIII* digestion, M: Marker, Pr: PCR product of 511 bp was used as the DNA probe.

QIAquick® PCR Purification Kit (QIAGEN®), adjusted to a concentration of 10 *n*g/*μl* and kept at −30°C until use. An alkaline phosphatase-labeled BLV *tax*-specific probe was prepared with the alkaline phosphatase direct labelling and detection system using CDP-*Star* (RPN3690, GE Healthcare, Piscataway, NJ, U.S.A.) [5]. Hybridization was performed with 4 *n*g/m*l* of the probe at 65°C. The signal was detected using high performance autoradiography film (Amersham Hyperfilm™-MP, GE Healthcare) for 45 min and followed by development.

The results of hybridization of the *tax* probe to *EcoRI* and *HindIII* fragments from EBL and non-EBL samples are shown in Figs. 1 and 2, respectively. There were several obvious bands in the 2 atypical and 3 typical EBL cattle (Fig. 1). In other words, the BLV-specific probe hybridized with 1 to 3 *EcoRI* and *HindIII* fragments from DNA obtained from peripheral blood of all EBL cattle. In contrast, the BLV *tax* probe hybridized to a large number of *EcoRI* and *HindIII* fragments in all 4 PL cattle (Fig. 2). The integration pattern of these cattle showed a 'smear' at more than 8,000 bp for *EcoRI* fragments and at more than 5,000 bp for *HindIII* fragments (Fig. 2). *EcoRI* fragments from three (BLV-3, 4 and 5) among 5 cattle positive for BLV infection without lymphocytosis showed a 'smear' pattern; however, samples from the remaining 2 cattle (BLV-1 and 2) showed no smear (Fig. 2). *HindIII* fragments from 2 (BLV-3 and 4) among 5 cattle positive for BLV infection without lymphocytosis showed also a 'smear' pattern (Fig. 2).

Since BLV randomly integrates into the genomic DNA of B-lymphocytes [19], polyclonal integration of BLV proviral DNA into the host genome should be observed in cattle without tumors, including clinically normal PL cattle or BLV carriers, as assessed by Southern blot [6, 8, 9]. Yet, once malignant transformation of lymphocytes occurs, B lymphocytes undergo monoclonal proliferation, and only DNA fragments of a specific length are amplified [6, 8, 9]. Thus, monoclonal proliferation of B-lymphocytes would result in one or several distinct bands in EBL samples. Such a monoclonal integration pattern can be used as evidence of neoplastic proliferation of B lymphocytes in EBL cases. Conversely, smear patterns found in PL cattle or some BLV infection cattle without lymphocytosis indicate the polyclonal existence of BLV-infected lymphocytes in these cattle. No bands were found in some BLV-positive cattle. It might reflect the less amount of BLV in the lymphocytes than detection level by the present method.

Our findings are similar to those of previous studies reporting monoclonal integration of the BLV provirus in malignant cells [8, 11]. However, in the present study, genomic DNA extracted from the peripheral blood of atypical EBL cases which did not show lymphadenopathy was analyzed to evaluate the utility of this diagnostic method for detecting EBL onset in difficult to diagnose cases. The present results suggest that detection of the monoclonal integration of BLV provirus into host genomic DNA by Southern blotting and hybridization could be used as a marker for leukosis onset in atypical EBL cases without lymphadenopathy. Detection of monoclonal integration of BLV provirus into host genomic

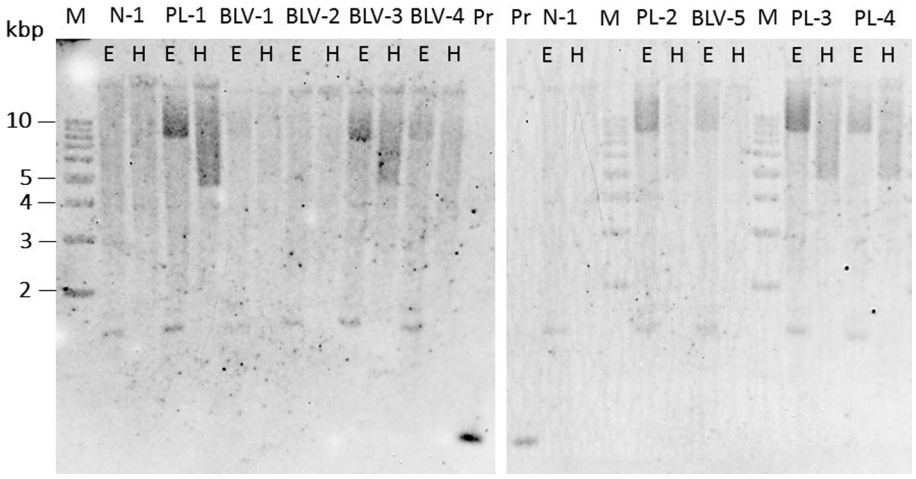

Fig. 2. Hybridization of the BLV *tax* probe to *EcoRI* and *HindIII* fragments in non-EBL cattle, including 4 cattle with persistent lymphocytosis (PL), 5 BLV infected cattle and 1 BLV-negative healthy cow. Integration patterns of the 5 PL cattle showed a 'smear' for both *EcoRI* and *HindIII* fragments. *EcoRI* fragments from 3 (BLV-3, 4 and 5) among 5 cattle with BLV infection without lymphocytosis showed a 'smear' pattern; however, samples from the remaining 2 cattle showed no smear. *HindIII* fragments from 2 (BLV-3 and 4) among 5 cattle positive for BLV infection without lymphocytosis showed also a 'smear' pattern. PL-1 to 4: Clinically normal cattle with persistent lymphocytosis and BLV infection. BLV-1 to 5: Clinically normal cattle with BLV infection but normal lymphocyte counts. N-1: Negative control without persistent lymphocytosis or BLV infection. E: *EcoRI* digestion, H: *HindIII* digestion, M: Marker, Pr: PCR product of 511 bp which was used as the DNA probe.

DNA may also serve as a marker for malignant transformation of circulating lymphocytes in PL cattle infected with BLV. This method may prove useful for detecting malignant cases among clinically normal cattle with BLV infection.

In the present study, it was demonstrated that detection of monoclonal integration of BLV provirus DNA can be used to detect EBL cattle without enlargement of surface lymph nodes. This suggested the monoclonal and malignant proliferation of lymphocytes in the peripheral blood of the cases, although no abnormal lymphocytes have been recorded by the microscopic examination. More EBL cases with no lymphocytosis and no lymphadenopathy should be analyzed to evaluate the sensitivity and specificity of this method described herein for the detection of malignant transformation.

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