Time course changes in peripheral B-cell clonality in a Japanese Black bull with enzootic bovine leukemia
ABSTRACT. A 38-month-old Japanese Black bull presenting with anorexia was given supportive treatment without improvement. Findings including bovine leukemia virus positivity and monoclonal B-cell proliferation strongly suggested the onset of enzootic bovine leukosis (EBL). Pathological findings confirmed the diagnosis of EBL. B-cell clonality were analyzed over time using pre-onset preserved genomic DNA at ages 6 months, 16 months, and 30 months. In the B-cell clonality analysis, two minor peaks at 140 and 220 bp were observed before onset, but another large peak at 175 bp appeared at the time of EBL diagnosis. Although the reason for the proliferation of an independent clone is unknown, detection of clonality abnormalities may lead to the detection of cattle at high risk of developing EBL.

KEYWORDS: B-cell clonality, enzootic bovine leukosis, Japanese Black, time course change
Enzootic bovine leukosis (EBL) is a malignant hematological tumor of cattle caused by bovine leukemia virus (BLV) infection [1]. It has been reported that 20-30% of cattle infected with BLV exhibit persistent lymphocytosis, and 2-3% of BLV-infected cattle develop EBL with an incubation period of more than 3 years [1]. EBL patients exhibit monoclonal proliferation of B lymphocytes in affected lymphatic organs or peripheral blood. A recent study reported that the clonality status of cattle with persistent leukosis (PL) was found to be polyclonal, oligoclonal, or minor-clonal [7]. Although cattle with oligoclonal proliferation are at higher risk for EBL, the time course changes in clonality of B lymphocytes in cattle from PL to the onset of EBL have not been examined. In the present study, we experienced a clinical case of EBL in a Japanese Black bull, in which B lymphocyte clonality had been analyzed over time at ages 6, 16, and 30 months, and at age 39 months, the time of diagnosed for EBL.

A 38-month-old Japanese Black bull presented with anorexia. Due to weak gastrointestinal motility, metoclopramide and dexamethasone were administered as symptomatic treatment on Day 1. Although supportive treatment including metoclopramide and fluid infusion had been given from Days 2 to 35, clinical signs gradually worsened and the patient showed difficulties with standing up. Although antibodies against BLV were detected, there was no swelling of surface lymph nodes. Complete blood counts on Day 36 revealed severe anemia (RBC: 278 x 10^4/µL, Hb: 4.4 g/dL, Ht: 11.9%), but there was no lymphocytosis (WBC: 8,240/µL, lymphocytes: 1,812/µL) in peripheral blood. Thirty-eight percent of peripheral lymphocytes were found to be atypical. Higher activities of onset markers for lymphoma, including thymidine kinase (38.9 U/L), total LDH (11,850 U/L), and LDH isozyme-2 (4,432 U/L) and -3 (2,406 U/L), were observed, suggesting the development of lymphoma [3,8].

B-cell clonality of peripheral blood on Day 36 was analyzed using two different clonality PCRs based on sequences of the variable region of the bovine immunoglobulin H chain, as reported previously [4,6]. Monoclonal proliferation of B-cells was observed by both methods (Figs.1D and 2D). This suggested that peripheral lymphocytes were B-cell lymphoma cells. Inverse PCR was also performed to demonstrate the monoclonal integration of BLV into the bovine genome [5]. Given the presence of a monoclonal peak (Fig. 3D), we speculated EBL caused by BLV.

The patient died on Day 39 and necropsy was immediately performed on the same day, revealing
multiple masses around the rumen and abomasum. The tumor also invaded the abomasum mucosa and an ulcer was identified. There was no lymphadenopathy in the thoracic cavity. Poor motility of the alimentary tract was considered to be due to the mass which formed in the rumen and abomasum. Histopathological findings of the abomasum mass confirmed neoplastic proliferation of lymphocytes (Fig. 4A). Immunohistochemistry using the Dako Envision+ System (Dako, Tokyo, Japan) revealed the neoplastic lymphocytes to be negative for CD3 (Dako) and positive for CD20 (Biocare Medical, Pacheco, CA, USA) and BLA36 (Dako), suggesting B-cell lymphoma (Figs. 4B-D).

Since the patient was a sire, blood had been regularly collected at least once a year. Genomic DNA samples extracted from peripheral blood at ages 6, 16, and 30 months were available. Real-time PCR for BLV revealed that the bull had been positive for BLV with high numbers of provirus copies since age 6 months (963 copies/10 ng DNA at age 6 months, 1,002 copies/10 ng DNA at age 16 months, and 379 copies/10 ng DNA at age 30 months). B-cell clonality of peripheral blood and clonality of the BLV integration site were also analyzed over time using pre-onset preserved genomic DNA. In the first B-cell clonality analysis, no monoclonal proliferation was detected before onset of EBL (Figs. 1A-C). In the second clonal PCR, abnormalities were detected in B-cell clonality from age 6 months. There were two small peaks at 140 and 220 bp in addition to the polyclonal curve at ages 6 and 16 months, and a peak at 140 bp at age 30 months (Figs. 2A-C). In addition to the two small peaks at 140 and 220 bp, a peak at 175 bp appeared at the time of diagnosed for EBL.

Time course changes of the BLV integration site were also analyzed (Fig. 3). A peak at 800 bp was dominant, but there were more smaller peaks observed since age 6 months. This oligoclonal proliferation pattern was also observed at age 16 and 30 months. However, at age of 39 month, a monoclonal peak at 2000 bp appeared (Fig. 3C) and the smaller peaks disappeared. Although the actual time could not be determined, the change from oligoclonal to monoclonal proliferation should have occurred between ages 30 and 39 months in the present case.

The present case was thought to be a case of atypical EBL because there was no swelling of surface lymph nodes and no lymphocytosis in peripheral blood. In such cases, it would be difficult to definitively diagnose EBL without necropsy. However, thymidine kinase and LDH activities, which are markers for
the onset of lymphoma [3, 8], were very high and predicted the onset of lymphoma. The usefulness of these biomarkers was reconfirmed in this case. In addition, two B-cell clonal PCRs based on sequences of the variable region of the bovine immunoglobulin H chain revealed monoclonal proliferation of peripheral B-cells at the time of onset. These results suggest that clonal PCR is useful for the diagnosis of atypical lymphoma in cases without lymphadenopathy and lymphocytosis. Inverse PCR detects monoclonal integration of BLV and is used to determine whether BLV is involved in lymphoma cases.

The present bull was a very rare clinical case of EBL in which clonal changes were followed-up over time using peripheral blood that had been stored regularly prior to onset. Oligo- or minor-clonal proliferation were observed before the onset of EBL in the present case. Our previous study reported that incidence of EBL onset within 1 year in cattle with oligo- or minor-clonal was higher than in cattle with polyclonal proliferation [7]. Human T-cell leukemia virus-1 (HTLV-1), which is closely related to BLV, is a causative agent of adult T-cell leukemia (ATL) in humans. Clonality abnormalities of HTLV-1 infected T-cells are known to be a risk indicator for the development and prognosis of ATL [2]. Detection of clonality abnormalities may lead to the detection of cattle infected with BLV at high risk of developing EBL.

Although clonality abnormalities such as oligoclonal and minor-clonal proliferation were observed before the onset of EBL, another clonal peak appeared at the time of diagnosed for EBL in our patient. The reason for the proliferation of an independent clone and whether the tumorigenic clone existed in pre-onset of EBL are unclear. Further investigation is needed to clarify these points by analyzing B-cell clonality changes in peripheral blood more frequently before and after onset of EBL.

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Fig. 1. Time course changes in peripheral B-cell clonality as assessed by PCR based on sequences of the variable region of the bovine immunoglobulin H chain [4]. (A) Age 6 months, (B) age 16 months, (C) age 30 months, and (D) age 39 months at the time of diagnosed for enzootic bovine leukosis (EBL). There is a minor peak at 140 bp and a polyclonal peak prior to EBL onset, while a monoclonal peak at 220 bp was detected at age 39 months (arrow).
Fig. 2. Time course changes in peripheral B-cell clonality as assessed by PCR based on sequences of the variable region of the bovine immunoglobulin H chain [6]. (A) Age 6 months, (B) age 16 months, (C) age 30 months, and (D) age 39 months at the time diagnosed for enzootic bovine leukosis (EBL). In addition to a polyclonal pattern, one or two minor peaks at 140 and 220 bp were observed before EBL onset, while a monoclonal peak at 175 bp was detected at age 39 months (arrow).
Fig. 3. Time course changes in clonality of bovine leukemia virus integration sites as assessed by inverse PCR [5]. A peak at 800 bp was dominant (black arrow), but smaller peaks were also observed since age 6 months (A). This oligoclonal proliferation pattern was also observed at ages 16 and 30 months (B and C). However, a very strong monoclonal peak (white arrow) appeared at 2000 bp (D) and the smaller peaks disappeared at the time of diagnosed for enzootic bovine leukosis. M: Marker.
Fig. 4. Histopathologically, the abomasum mass consists of diffuse proliferation of neoplastic lymphocytes (A). Hematoxylin and eosin staining. Immunohistochemically, the neoplastic lymphocytes are negative for CD3 (B) and positive for CD20 (C) and BLA36 (D), suggesting B-cell lymphoma. Bar=50 μm.