Diagnosis and Early Prediction of Lymphoma Using High-Throughput Clonality Analysis of Bovine Leukemia Virus-Infected Cells

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ABSTRACT  Bovine leukemia virus (BLV), a retrovirus, infects B cells of ruminants and is integrated into the host genome as a provirus for lifelong infection. After a long latent period, 1% to 5% of BLV-infected cattle develop aggressive lymphoma, enzootic bovine leukemia (EBL). Since the clonal expansion of BLV-infected cells is essential for the development of EBL, the clonality of proviral integration sites could be a molecular marker for diagnosis and early prediction of EBL. Recently, we developed Rapid Amplification of the Integration Site without Interference by Genomic DNA Contamination (RAISING) and an analysis software of clonality value (CLOVA) to analyze the clonality of transgene-integrated cells. RAISING-CLOVA is capable of assessing the risk of adult T-cell leukemia/lymphoma development in human T-cell leukemia virus-I-infected individuals through the clonality analysis of proviral integration sites. Thus, we herein examined the performance of RAISING-CLOVA for the clonality analysis of BLV-infected cells and conducted a comprehensive clonality analysis by RAISING-CLOVA in EBL and non-EBL cattle. RAISING-CLOVA targeting BLV was a highly accurate and reproducible method for measuring the clonality value. The comprehensive clonality analysis successfully distinguished EBL from non-EBL specimens with high sensitivity and specificity. A longitudinal clonality analysis in BLV-infected sheep, an experimental model of lymphoma, also confirmed the effectiveness of RAISING-CLOVA for early detection of EBL development. Therefore, our study emphasizes the usefulness of RAISING-CLOVA as a routine clinical test for monitoring virus-related cancers.

IMPORTANCE  Bovine leukemia virus (BLV) infection causes aggressive B-cell lymphoma in cattle and sheep. The virus has spread to farms around the world, causing significant economic damage to the livestock industry. Thus, the identification of high-risk asymptomatic cattle before they develop lymphoma can be effective in reducing the economic damage. Clonal expansion of BLV-infected cells is a promising marker for the development of lymphoma. Recently, we have developed a high-throughput method to amplify random integration sites of transgenes in host genomes and analyze their clonality, named as RAISING-CLOVA. As a new application of our technology, in this study, we demonstrate the value of the RAISING-CLOVA method for the diagnosis and early prediction of lymphoma development by BLV infection in cattle. RAISING-CLOVA is a reliable technology for
monitoring the clonality of BLV-infected cells and would contribute to reduce the economic losses by EBL development.

**KEYWORDS** bovine leukemia virus, cattle, clinical diagnosis, high-throughput analysis, lymphoma, retrovirus, sheep, clonality

Enzootic bovine leukosis (EBL) is a B-cell lymphoma or leukemia in cattle caused by infection with bovine leukemia virus (BLV). BLV is a member of the genus *Deltaretrovirus*, subfamily *Orthoretrovirinae*, family *Retroviridae*, and is genetically related to human T cell leukemia virus type 1 (HTLV-1) (1). BLV infects B cells of ruminants, and its viral RNA is reverse-transcribed into double-stranded DNA and then integrated into the host genome as a provirus. Most infected cattle are asymptomatic (aleukemic; AL) throughout their life span. About 30% of infected cattle develop persistent lymphocytosis (PL), an abnormal proliferation of BLV-infected B cells. EBL occurs in 1% to 5% of BLV-infected cattle, which present with B-cell malignant lymphoma in lymph nodes and various other organs, leading to a poor prognosis and death (2).

BLV has been eradicated in some European and Oceanian countries through the identification and culling of BLV-infected cattle but is still endemic worldwide (3). The incidence of BLV infection has been increasing during recent decades in Japan; a nationwide survey of cattle conducted from 2009 to 2011 confirmed BLV infection in 40.9% of dairy cattle and 28.7% of beef cattle (4). The countermeasures against BLV are urgently needed, but there is no commercially available vaccine or therapeutic drug against BLV. Therefore, the herd management by detection, quarantine, and culling of infected cattle is the most effective method of controlling BLV infection. However, this approach is difficult to implement in areas of endemicity with large numbers of infected cattle.

In addition, the use of cattle bearing leukemia for milk or meat production is prohibited in Japan, and they must be immediately slaughtered, which causes significant production losses to farmers. To reduce the economic damage and food loss caused by EBL in such areas of endemicity, it is effective to identify cattle at high risk of EBL development in advance.

During the development of EBL, one or few clones of BLV-infected cells undergo clonal expansion. These malignant cells possess identical integration sites of BLV provirus. Therefore, the clonality of proviral integration sites in BLV-infected cells has been considered a useful marker to diagnose the onset of lymphoma (5–8). Several methods have been recently developed to analyze transgene integration sites using high-throughput sequencing (HTS), such as ligation-mediated PCR (5, 9), target capture sequencing (7, 10, 11), inverse PCR (12), linear amplification-mediated PCR (LAM-PCR) (6), and nonrestrictive LAM-PCR (13). However, it would be difficult to analyze multiple specimens sensitively, rapidly, and inexpensively by these current methods. Therefore, there is a need to develop a high-throughput method that overcomes the problems of current methods and that can be applied in clinical testing for BLV clonality analysis.

Recently, we developed Rapid Amplification of Integration Sites without Interference by Genomic DNA Contamination (RAISING) and an analysis software of clonality value (CLOVA) as a highly sensitive, rapid, inexpensive, and high-throughput method to amplify and analyze random integration sites of transgenes in host genomes (14). RAISING and CLOVA were originally developed for analyzing the clonality value (Cv) of provirus integration sites in HTLV-1-infected cells (14, 15). The Cv analyzed by RAISING-CLOVA using the blood specimens of HTLV-1 carriers is an effective marker for the prediction of the risk of adult T cell leukemia/lymphoma (ATL) development (14).

BLV is considered to utilize similar mechanisms for proviral integration and tumorigenesis as HTLV-1 (9, 16). In our previous study, the proviral integration sites of BLV-infected cells were successfully amplified by RAISING using one specimen of each AL and EBL cattle (14). Sanger sequencing and HTS confirmed the clonal expansion of BLV-infected cells only in the EBL specimen, even though the number of tested specimens was quite limited (14). The clonality analysis of BLV-infected cells by RAISING-CLOVA could be an effective method for the diagnosis and prediction of the EBL onset in cattle. Hence, in this study, we performed the comprehensive clonality analysis of BLV-infected cells using RAISING-CLOVA in EBL and
non-EBL cattle and examined its performance in the diagnosis and prediction of the onset of lymphoma in cattle and sheep.

RESULTS

Performance of clonality analysis by RAISING-CLOVA targeting BLV. To examine the detection limit of RAISING targeting BLV, RAISING was performed using a series of DNA samples from mixtures of different percentages of a BLV-infected B-cell line (BL3.1) and a BLV-uninfected cell line (BTL26) and the amplified products were analyzed by Sanger sequencing. BL3.1 harbors multiple copies of BLV provirus in the genome (8) and proviral load (PVL) of BL3.1 was 240 copy/100 cells. Thus, DNA samples extracted from specimens, including 0.0001% to 100% BL3.1 contain different PVL ranging from 0.0024 to 240 copy/100 cells. The amplified fragments were observed in the electrophoresis and the combined sequences of BLV provirus and host genome were detected in the DNA samples containing 0.012 to 240 copy/100 cells of provirus (Fig. 1A and Fig. S1 in the supplemental material). The detection limit for RAISING targeting BLV was 0.012 copy/100 cells in PVL, which is comparable with that for RAISING targeting HTLV-1 (0.032% in HTLV-1 PVL) (14). These results suggest that RAISING targeting BLV is sensitive enough to detect proviral insertion sites, even in specimens with low PVL.

Our previous study has shown that RAISING-CLOVA targeting HTLV-1 fails to accurately measure Cv in samples with PVL less than 0.5% (14). Here, we examined the sensitivity of clonality analysis by RAISING-CLOVA targeting BLV. The Cv of BL3.1 was 0.56 when measured using the specimen with PVL 240 copy/100 cells (Fig. 1B and Fig. S1 in the supplemental material). The values were comparable (Cv: 0.55 to 0.60) to the original Cv of BL3.1 when measured using the specimens with PVL 1.2–120 copy/100 cells but were deviated (Cv: 0.66 to 0.78) from the original one in the specimens with PVL lower than 0.24 copy/100 cells (Fig. 1B and Fig. S1 in the supplemental material). This result indicates that RAISING-CLOVA targeting BLV can accurately measure Cv when the PVL is at least 1.2 copy/100 cells and is feasible enough for the analysis of most BLV-infected specimens.

To further examine the performance and accuracy of the clonality analysis by RAISING-CLOVA, we tested DNA samples obtained from BLV-infected cattle with or without EBL. Representative results of RAISING-CLOVA in blood specimens of BLV-infected cattle with different values of Cv are shown in Fig. 1C. The Cv reflected the intensity of the sequence signal peaks of integration sites at the host genome (Fig. 1C). These results are consistent with our previous results of RAISING-CLOVA using specimens of HTLV-1-infected patients as well as a preliminary data using specimens of BLV-infected cattle (14). Furthermore, the clonality analyses of the identical BLV-infected specimens (n = 32) in the two different laboratories showed a high interrater agreement in Cv (Fig. 1D). These results indicate that RAISING-CLOVA is highly accurate and reproducible method for measuring the clonality of BLV-infected cells.

Comprehensive clonality analysis of BLV-infected cells in EBL and non-EBL cattle. The clonality analysis by RAISING-CLOVA was performed on peripheral blood samples from AL (n = 107), PL (n = 79), and EBL cattle (n = 101), and tissue samples, including tumors from EBL cattle (n = 169) collected from farms throughout Japan (Tables S4 and 5 in the supplemental material). In addition, BLV PVL was also measured in these samples, because a previous study proposed that PVL is a candidate marker for EBL diagnosis (17). Representative results of RAISING-CLOVA in EBL and non-EBL blood specimens are shown in Fig. 2A. In an EBL cattle (EBL015), Sanger sequencing analysis detected monoclonal patterns of proviral integration in peripheral blood and tumor specimens and their Cv were calculated as 1.00 (Fig. 2A), indicating clonal expansion of BLV-infected cells. In contrast, in non-EBL cattle (AL002 and PL002), the sequencing analysis detected polyclonal patterns of proviral integrations with Cv 0.09 and 0.06, respectively (Fig. 2A). Among the blood samples, EBL showed significantly higher Cv than AL and PL (median: 0.63, 0.09, and 0.08, respectively) (Fig. 2B). In contrast, there was no significant difference in the PVL of blood between PL and EBL (median: 35.10 and 25.78 copy/100 cells, respectively), although tumors of EBL showed higher PVL (median: 59.92 copy/100 cells) than blood samples at all disease stages (Fig. 2C). In addition, the Cv of tumors in EBL was higher (median: 0.79) than that of their blood (median: 0.63).
FIG 1 Performance of BLV clonality analysis by RAISING-CLOVA. (A, B) Detection limit of RAISING targeting BLV and sensitivity of its clonality analysis by RAISING-CLOVA. RAISING-CLOVA was performed using genomic DNA from the mixture of the BLV-infected cell line BL3.1 with the BLV-uninfected cell line. (Continued on next page)
(Fig. 2B). Tumors of EBL were observed in a variety of tissues in cattle, including multiple lymph nodes and nonlymphoid tissues such as heart, kidney, uterus, and digestive and respiratory organs. There were no significant differences in the Cv and PVL of tumors from lymphoid and nonlymphoid tissues in EBL (Fig. 2D and E). Furthermore, identical integration sites of BLV provirus were detected by Sangar sequencing among blood and tumor samples from same EBL cattle, even in the blood specimens with low Cv (EBL049 and EBL174) (Fig. S2). These results indicate that the Cv of blood samples analyzed by RAISING-CLOVA is an effective marker for distinguishing EBL from non-EBL cattle.

**Clinical utility of clonality analysis by RAISING-CLOVA targeting BLV.** To determine whether the Cv or PVL of BLV-infected cells was valuable for the diagnosis of EBL, we performed receiver operating characteristic (ROC) analysis and examined the specificity and sensitivity of EBL diagnosis by Cv and PVL in the blood samples. The area under the ROC curve (AUC) for Cv (0.9368) was higher than that for PVL (0.5759) in the blood samples of EBL and non-EBL cattle (Fig. 3A and B). The cutoff value of Cv in blood for EBL diagnosis was 0.17, which could distinguish EBL cases with 87.1% sensitivity and 93.0% specificity (Fig. 3A and C).

Out of 101 blood specimens of EBL, the Cv less than 0.17 was detected in 13 specimens (12.9%) (Fig. 3D). These animals showed clonal expansion of malignant cells in tumors, but not in blood. In addition, 13 cases (7.0%) of AL and PL cattle were identified with the Cv more than 0.17 in blood (Fig. 3D). Blood samples may also be suitable for follow-up and prognostic studies of non-EBL cattle with intermediate Cv. On the other hand, the cutoff value of PVL in blood was 32.10 copy/100 cells, which was not a suitable marker, because more than half of the EBL specimens had lower values than the cutoff (Fig. 3B and D). Thus, these experiments indicate that Cv is a better diagnostic marker for EBL compared to PVL.

**Longitudinal clonality analysis of experimental lymphoma model of sheep.** Sheep infected with BLV develop lymphoma at higher frequencies after shorter latency periods than cattle (2). To further investigate the usefulness of Cv as a predictive marker for the development of EBL, longitudinal clonality analysis was conducted in an experimental infection model of sheep with BLV (Table S6). In BLV-challenged sheep, Cv remained low immediately after infection, but increased before or at the onset of lymphoma (Fig. 4A). In three of the four tested animals (24, 93, and 114), Cv peaked earlier than PVL in blood (Fig. 4A). Sequence analysis of the integration sites in blood and tumor tissues also revealed that the identical integration sites were detected before and at the onset of lymphoma in all tested sheep (Fig. 4B). These results indicate that clonality analysis by RAISING-CLOVA is a promising method for early prediction of lymphoma onset in BLV infection.

**DISCUSSION**

In the cattle industry, BLV infection is widespread in all parts of the world except some of the European and Oceanian countries (3) and causes major economic losses in the production of milk and beef (18, 19). However, there is no effective treatment or vaccination to control BLV infection. In countries where BLV is endemic such as Japan and American countries (3, 4), the control measure of eradicating BLV-infected cattle is completely impractical. Therefore, the development of a novel method for early prediction of tumor development at the carrier stage would contribute to the reduction of economic losses in livestock production. In this study, we applied a novel molecular method called RAISING with the CLOVA software (14) to...
Comprehensive clonality analysis by RAISING-CLOVA targeting BLV using specimens of EBL and non-EBL cattle. (A–E) RAISING-CLOVA was performed using genomic DNA of blood samples of AL (n = 107), PL (n = 79), and EBL cattle (n = 101) and tumor samples of EBL cattle (n = 169).

FIG 2 Comprehensive clonality analysis by RAISING-CLOVA targeting BLV using specimens of EBL and non-EBL cattle. (A–E) RAISING-CLOVA was performed using genomic DNA of blood samples of AL (n = 107), PL (n = 79), and EBL cattle (n = 101) and tumor samples of EBL cattle (n = 169).
amplify BLV proviral integration site in host genome and analyze the clonality of BLV-infected cells. We further examined the usefulness of the clonality analysis by RAISING-CLOVA for diagnosis and early prediction of EBL development.

For BLV clonality analysis, several molecular methods have been developed to analyze proviral integration sites of BLV-infected cells, including ligation-mediated PCR (5), LAM-PCR (6), target capture sequencing (7), and inverse PCR (8, 12). Most of these methods require HTS analysis for the detection of integration sites (5–7, 12), but the high cost of the analysis makes it unsuitable for clinical diagnosis with large numbers of samples. Additionally, these current methods utilize restriction enzymes or ultrasound sonication for DNA fragmentation, which raises concerns about the bias of amplification or detection, causing their poor sensitivity and reproducibility.

RAISING is a highly sensitive, highly accurate, rapid, inexpensive, and high-throughput method to overcome the problems of conventional methods (14). In this study, we analyzed Cv of BLV-infected cells in EBL and non-EBL cattle using RAISING-CLOVA and found that Cv discriminated between nonmalignant and malignant samples successfully but BLV PVL did not. PVL has been considered to be a significant marker in EBL diagnosis (17). However, the sensitivity and specificity of EBL diagnosis by PVL in blood samples was very poor in this study, which is consistent with another previous study (20). Although elevation of PVL appear to be a necessary step in the development of EBL, it is not a suitable diagnostic marker for the development and early diagnosis of EBL. The clonality analysis by RAISING-CLOVA should be conducted with another cohort of clinical samples to confirm the reproducibility of the present analysis.

Among EBL cases analyzed in this study, 13 specimens (12.9%) did not show tumorigenesis in peripheral blood, a finding which is consistent with our previous report (21). In EBL cattle, lymphocytosis and the presence of atypical lymphocytes are reportedly observed in peripheral blood of EBL cattle, but these clinical signs do not appear to be relevant in all cases. However, more detailed EBL typing may be possible by examining the clonality of tumor cells in the blood and tissues of larger numbers of EBL cattle. In addition, recent studies suggest that tumor cells of EBL sometimes harbor defective proviruses (7, 12). EBL diagnosis using qPCR targeting provirus would miss those defective cases. In this study, several tumor specimens with proviruses deficient in the pol gene, which was the target of qPCR in this study, were identified in EBL cattle (data not shown). The 3’ LTR and its upstream regions are less likely to be deficient in the provirus of EBL tumors (7, 12) and are an optimal target of gene specific primers for RAISING.

In BLV infection, antisense transcripts, called AS, were found to be constantly expressed in EBL tumors through promoter activation of the 3’ LTR region (22). A recent study reported that activation of antisense transcription originating from the 3’ LTR forms a chimeric transcript of the AS gene and the host driver gene upstream of the provirus, resulting in enhanced transcription of driver genes during BLV infection and following tumorigenesis (10). Therefore, it is important to analyze the integration site of BLV provirus in the bovine genome in order to investigate its contribution to tumorigenesis of EBL. Further studies are warranted to address this issue by HTS analysis of the amplicon of RAISING in non-EBL and EBL cattle.

To further validate the usefulness of clonality analysis by RAISING-CLOVA for a risk assessment of EBL development, a comprehensive follow-up study of non-EBL cattle is required in a clinical setting, focusing on carrier animals showed higher Cv in the screening test. Previous studies have demonstrated that clonality analysis of HTLV-1-infected carriers showed that the Cv of infected cells increased earlier than the increase in peripheral blood provirus levels before the onset of ATL, suggesting the applicability of this method to the risk assessment of ATL (14, 15). In this study, similar results were confirmed in a sheep lymphoma model. Sheep
FIG 3 Clinical utility of BLV clonality analysis by RAISING-CLOVA using blood and tumor samples. (A, B) ROC analysis using Cv (A) and PVL (B) of blood samples to distinguish EBL in the blood samples. AUC, area under the curve; CI, confidence interval. (C) Frequencies (Continued on next page)
can be a good model for validating the usefulness of this method, because they develop lymphoma at high frequencies and with shorter period compared to cattle (2). We will also test additional sheep samples for further validation. Taken together, BLV clonality analysis by RAISING-CLOVA is a potential method for selection of high-risk animals among BLV-infected carriers, which would contribute to the reduction of economic damage and food loss caused by EBL in the areas of endemicity.

MATERIALS AND METHODS

Blood and tumor samples. Peripheral blood of BLV-infected cattle (Holstein, Japanese Black, or crossbreds) was collected from dairy and beef farms in Japan between 2017 and 2022. Peripheral blood, lymph nodes, spleen, and various organs were also collected from cattle diagnosed with lymphoma (Holstein, Japanese Black, or crossbreds) at Livestock Hygiene Service Centers and Meat Hygiene Inspection Centers in Japan between 2013 and 2022. The blood and tumor samples were kept refrigerated until cell separation. The animal experiments were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval number 17-0024). Verbal informed consent was obtained from the owners for the participation of their animals in this study. Experimental infection of BLV in sheep was conducted at the Research Farm in the Field Science Center, Faculty of Agriculture, Iwate University. Sheep (Corriedale or Suffolk, 3 months old) were intraperitoneally inoculated with 3.0 × 10⁷ cells of BLV-infected leukocytes isolated from BLV-infected cattle. After the viral challenge, peripheral blood was collected from the BLV-challenged sheep. The procedures were approved by the Iwate University Animal Care and Use Committee (approval no. A201703). All of the animal experiments in this study were followed by the Recommendations in the ARRIVE guidelines (23).

Cell isolation and cultivation of cell line. Peripheral blood mononuclear cells (PBMCs) were separated from blood samples by density gradient centrifugation using Percoll (GE Healthcare, Chicago, IL, USA). Whole blood was lysed with ACK lysing buffer to separate white blood cells. Separated cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4) and filtered through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA). Tissue specimens were shredded with scissors and filtered using a 40 μm or 100 μm cell strainer (BD Biosciences) to obtain cell suspensions and washed twice with PBS. Cells were stained with Trypan Blue Stain (Thermo Fisher Scientific, Waltham, MA, USA) and the number of viable cells was measured using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific). Cells were either used immediately or frozen at −80°C until used in experiments.

BLV-infected B-cell line BL3.1 (24) and BLV-uninfected T-cell line BTL26 (25) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Thermo Fisher Scientific) at 37°C and 8% CO₂.

Preparation of genomic DNA. Genomic DNA from whole blood samples, PBMCs, and tissue specimens of cattle was extracted using Wizard Genomic DNA purification kits (Promega, Madison, WI, USA) or Quick-DNA Miniprep Kits (Zymo Research, Irvine, CA, USA). Genomic DNA from whole blood samples of sheep was extracted using MagDEA Dx SV (Precision System Science, Matsudo, Japan) with a magLEAD 12gC instrument (Precision System Science). Genomic DNA was extracted from mixtures of BL3.1 and BTL26 for a total of 1 × 10⁶ cells using Quick-DNA Miniprep Kits (Zymo Research). The DNA concentrations of the samples were measured by UV absorbance at 260 nm using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific). Agarose gel electrophoresis was performed to check for the degradation of each DNA sample.

Quantification of BLV PVL. The BLV pol gene was measured in the genomic DNA samples of blood and tissue samples of cattle using real-time PCR with a BLV Detection kit (TaKaRa Bio, Otsu, Japan) with a LightCycler 480 System II (Roche Diagnostics, Mannheim, Germany). A serial dilution series of the positive-control DNA for each kit were used to generate calibration curves to determine the copy number of the BLV provirus. Each DNA sample was tested in duplicate. For the blood and tumor samples of sheep, the BLV tax gene was measured in the DNA samples using real-time PCR, as described previously (26).

Diagnosis of BLV infection and EBL. BLV infection in cattle was confirmed by the detection of anti-BLV antibodies in plasma using a commercial ELISA kit (JNC, Tokyo, Japan) and by the detection of BLV provirus in DNA using real-time PCR, as described above. Samples tested positive for anti-BLV antibody and the provirus were diagnosed as “BLV-infected.” The number of lymphocytes in blood samples was counted using an automated hematology analyzer (Celltac; αi Nihon Kohden, Tokyo, Japan). BLV-infected cattle were classified as AL or PL based on the lymphocyte counts as follows: AL: < 8,000 cells/μL; PL: > 8,000 cells/μL. Blood and tissue samples from cattle with lymphoma were diagnosed as B-cell lymphoma based on immunophenotyping analysis by flow cytometry and/or B-cell clonality analysis by PCR targeting bovine immunoglobulin heavy chain (21) combined with the quantification of BLV provirus by real-time PCR, as described above.

Amplification of integration sites of BLV provirus by RAISING. The integration sites of BLV provirus were amplified by RAISING as previously described (14), with some modifications. The primers and reagents used in each step are shown in Tables S1 and 2 in the supplemental material. The reaction conditions of each step are shown in Table S3. Briefly, single-stranded DNA (ssDNA) of the 3’ LTR region of the BLV provirus and
FIG 4 Longitudinal clonality analysis of BLV-infected sheep with lymphoma. (A, B) RAISING-CLOVA was performed using genomic DNA of blood and tumor samples of BLV-challenged sheep (n = 4). (A) Kinetics of BLV PVL (blue circle) and Cv (orange diamond) in blood samples of BLV-infected sheep from viral challenge to lymphoma onset. (B) Sequence analysis of the proviral integration sites in blood and/or tumor samples of the challenged sheep.
the downstream region of the host genome was synthesized from the extracted genomic DNA using the primer BLV-F1 and KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan). The synthesized ssDNA was purified using a Monarch PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA, USA) and was eluted in ultrapure water. Then, poly(A) and poly(G) tails were added at the 3’ end of the purified ssDNA by terminal transferase (New England Biolabs). The double-stranded DNA was then synthesized and amplified by PCR from the poly(A)-tailed ssDNA using the primers BLV-F2 and NV-oligo-dT-ADP1 and Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs). The second PCR was performed using diluted PCR products, the primers BLV-F3 and ADP1-HTS-R1, and KOD-Plus-Neo DNA polymerase (Toyobo).

Sequencing and clonality analysis using CLOVA. The products of the second PCR were purified using AMPure XP (Beckman Coulter, Fullerton, CA, USA) and analyzed using Sanger sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on a 3130xl or 3730xl DNA Analyzer (Thermo Fisher Scientific). Clonality analysis of BLV-infected cells was performed using the sequencing signal data of each sample with a CLOVA software (14), an R program that automatically analyzes the CV of transgene-integrated cells, by dividing the average of signal peak area values of 20 nucleotides at 5’ terminal of host genome sequence of the dominant clone by that at 3’ terminal of BLV proviral sequence.

Statistical analysis. Significant differences among multiple groups were identified using Kruskal-Wallis one-way analysis of variance followed by Dunn’s test. Association of two values were tested using ROC curve analyses were performed to determine the optimal cutoff values, where sensitivity approximates specificity for each risk factor. All statistical tests were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at \( P < 0.05 \).

Data availability. All data sets and an uncropped gel image analyzed during the current study are provided in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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T.O., S.K., M.S., and K.O. designed the research. T.O., H.S., M.S., T.M., N.N., and S.Y. performed the experiments. M.S., T.M., N.N., S.Y., and K.M. provided intellectual input, field samples, laboratory materials, reagents, and/or analytic tools. T.O., H.S., S.K., M.S., T.M., N.N., S.Y., K.M., N.M., S.M., and K.O. analyzed and interpreted the data. T.O. and H.S. wrote the manuscript. T.O., S.K., M.S., S.Y., K.M., and K.O. revised the manuscript. All authors read and approved the final manuscript.

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