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Variations in the viral genome and biological properties of bovine leukemia virus wild-type strains

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ABSTRACT

Bovine leukemia virus (BLV) is the etiological agent of enzootic bovine leukosis (EBL), which causes enormous economic losses in the livestock industry worldwide. To reduce the economic loss caused by BLV infection, it is important to clarify the characters associated with BLV transmissibility and pathogenesis in cattle. In this study, we focused on viral characters and examined spontaneous mutations in the virus and viral properties by analyses of whole genome sequences and BLV molecular clones derived from cows with and without EBL. Genomic analysis indicated that all 28 strains harbored limited genetic variations but no deletion mutations that allowed classification into three groups (A, B, and C), except for one strain. Some nucleotide/amino acid substitutions were specific to a particular group. On the other hand, these genetic variations were not associated with the host bovine leukocyte antigen-DRB3 allele, which is known to be related to BLV pathogenesis. The viral replication activity *in vitro* was high, moderate, and low in groups A, B, and C, respectively. In addition, the proviral load, which is related to BLV transmissibility and pathogenesis, was high in cows infected with group A strains and low in those infected with group B/C strains. Therefore, these results suggest that limited genetic variations could affect viral properties relating to BLV transmissibility and pathogenesis.

1. Introduction

Bovine leukemia virus (BLV), which belongs to the family *Retroviridae* genus *Deltaretrovirus*, is the etiologic agent of enzootic bovine leukosis (EBL), which is a lethal infectious disease of cattle. The BLV infection results not only in EBL development but also in reductions in lifetime milk production, reproductive efficiency, and lifespan (Brenner et al., 1989; Nekouei et al., 2016; Polat et al., 2017b; Schwartz and Levy, 1994). In addition, the prevalence of BLV infection is high in several regions worldwide (Ott et al., 2003) and is thus responsible for economic losses throughout the livestock industry. However, BLV in infected cows cannot be technically eliminated because BLV integrates into the host genomic DNA of peripheral blood cells as a provirus (Murakami et al., 2011). Therefore, the prevention of BLV infection spread and EBL development would help in reducing the economic losses due to BLV infection in the livestock industry worldwide.

Useful information for controlling of infectious diseases can be obtained by analyzing factors involved in transmissibility and pathogenesis. Previous studies have demonstrated that the host factors, such as bovine leukocyte antigen (BoLA) and immunoreaction (Aida et al., 2013; Kabeya et al., 2001; Lewin and Bernoco, 1986; Lewin et al., 1988; Miyasaka et al., 2013; Murakami et al., 2004; Nishimori et al., 2017), affect BLV transmissibility and pathogenesis of BLV, whereas induced mutations in BLV genome regions can also have an effect (Florins et al.,

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2006, 2007; Gillet et al., 2016; Inoue et al., 2011, 2013; Watanabe et al., 2015). The BLV genome is relatively well conserved and the genetic variations are limited (Mansky and Temin, 1994), while the analyses of host factors are more advanced than those of viral factors. The changes in viral properties resulting from mutations that affect transmissibility and pathogenesis have mainly been analyzed by inducing mutations. Thus, it remains ambiguous whether spontaneous variations in the BLV genome affect viral properties that are related to viral transmissibility and pathogenesis.

The BLV genome contains a variety of functional genes and regions. The BLV proviral genome encodes a long terminal repeat (LTR) at both the 5′ and 3′ termini, and the *gag-pro-pol*, *env,* and nonstructural genes are encoded between the two LTRs. The nonstructural genes *AS1*, *R3*, *G4*, *tax*, and *rex* are reportedly encoded within the pX region, which is located between the *env* and 3′ LTR (Aida et al., 2013; Durkin et al., 2016). In addition, five micro RNAs (miRNAs) are encoded between the *env* and *R3* genes (Kincaid et al., 2012; Rosewick et al., 2013), and the noncoding RNA, AS2, is encoded on the minus strand of the *gag-pro-pol* gene (Durkin et al., 2016). In our previous study, a small spontaneous deletion mutation in the *G4* gene resulted in low virus production and static proviral load (PVL), which is closely related to transmissibility and pathogenesis (Jimba et al., 2010; Juliarena et al., 2016; Ohno et al., 2015; Somura et al., 2014), in a BLV-infected cow (Murakami et al., 2016). In addition, BLV strains harboring spontaneous nonsense mutation were identified in BLV-infected cows, and spontaneous mutations in limited regions may be related to BLV pathogenesis (de Brogniez et al., 2015; Inoue et al., 2011, 2013; Matsumura et al., 2011; Moratorio et al., 2013; Watanabe et al., 2015; Willems et al., 2000). These previous reports suggest that these spontaneous limited variations in the BLV genome may have the potential to change viral properties relating to transmissibility and pathogenesis. Thus, we hypothesized that each wild-type strain harboring spontaneous nucleotide substitutions in BLV genome has different viral properties. In this study, we examined the association between genetic variations over the whole BLV genome and viral properties based on virus activity *in vitro* and the PVL in BLV-infected cows.

2. Materials and methods

2.1. Collection of samples

Blood samples were collected from Holstein–Friesian cows from dairy farms in Japan. Thirteen tumor samples were provided by a meat inspection center and other farms in Japan. Detailed information for each sample is provided in Table 1.

2.2. Extraction of DNA from blood and tumor samples

Genomic DNA was extracted from whole blood and tumor samples using the Wizard genomic DNA purification kit (Promega Corporation, Madison, WI, USA) and the Get *pure*DNA Kit-Cell, Tissue (Dojindo Molecular Technologies, Rockville, MD, USA), respectively, according to the manufacturers' instructions.

2.3. Detection of BLV infection in cows

These sera isolated from blood and genomic DNA extracted from tumors were subjected to the agar gel immunodiffusion (AGID) test and polymerase chain reaction (PCR) analysis, respectively. The AGID test was performed as described previously (Murakami et al., 2016). Briefly, a gel with one central well and six surrounding wells was prepared. The BLV antigen and the positive reference serum were placed into the central well and into two symmetrical outer wells, respectively, whereas the serum samples, which were prepared by centrifugation of whole blood, were placed into the remaining four wells. After incubation in a humidified chamber at room temperature for 48 h, the presence of precipitation lines that indicate the presence of BLV-specific antibodies was determined by eye. For PCR, a partial *pol* gene from the BLV genome, which is highly conserved, was amplified using Go-Taq Green Master Mix (Promega Corporation), as described previously (Murakami et al., 2016).

2.4. Sequencing of the BLV proviral genome

BLV genomes were sequenced, as described previously (Murakami et al., 2016). The whole BLV genome was amplified using PrimeSTAR GXL polymerase (Takara Bio, Inc., Shiga, Japan) and primers (Supplementary Table S1). The PCR products from each sample were electrophoresed in 1% agarose gels and purified from the gels using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation). The purified PCR products were used as templates for sequencing. The sequences of the products were determined using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), according to the manufacturers' instructions. The whole BLV genome sequences of the 27 strains identified in this study were deposited in the GenBank database under the accession numbers AP018006 to AP018032.

2.5. Phylogenetic analysis

The genome sequences were aligned by ClustalW, and maximum-likelihood trees were constructed, using MEGA 7.0 software (Kumar et al., 2016), as described previously (Polat et al., 2016). The reliability of the phylogenetic relationships was evaluated using nonparametric bootstrap analysis with 1000 replicates. Partial sequences (400 bp) of the *env* gene and whole BLV genome were used because the method described by Rola-Luszczak et al. (2013) can correctly identify each BLV genotype using a short sequence of the *env* gene sequence.

2.6. Alignment of amino acid and nucleotide sequences

Editing and alignment of nucleotide and amino acid sequences were performed using UGENE (Okonechnikov et al., 2012) and MEGA 7.0 software (Kumar et al., 2016).

2.7. Typing of the BoLA-DRB3 gene

Polymorphisms of the *BoLA-DRB3* gene in BLV-infected cows were identified using a PCR sequence-based typing method as described previously (Takeshima et al., 2011). Briefly, the partial *BoLA-DRB3* gene fragments of the cows listed in Table 1 were amplified by PCR. The sequences of the amplified PCR products were determined by direct sequencing. Based on the sequence data, *BoLA-DRB3* alleles were identified using ASSIGN 500ATF software (Conexio Genomics PTY, Fremantle, Australia).

2.8. Construction of BLV molecular clones and plasmids

Cloning of the whole BLV genome was performed as described previously (Murakami et al., 2016). Briefly, the BLV proviral genome was amplified by PCR using PrimeSTAR GXL DNA polymerase (Takara Bio, Inc.) and cloned into the pSMART LC Amp vector (Lucigen Corporation, Milwaukie, WI, USA). The BLV molecular clones were transfected into *Escherichia coli* (*E. coli*) strain Stbl3 cells. The constructed molecular clones were confirmed by sequencing using primers described in Supplementary Table S2.

The BLV U3 promoter sequence and firefly luciferase sequences were amplified by PCR using the genomic DNA of FLK-BLV and the pCMV-Luc plasmid vector (Promega), respectively, as a template DNA. The amplified U3 promoter sequence was replaced with the EF1α promoter region of pBApo-EF1α-Pur (Takara Bio, Shiga, Japan), and the amplified firefly luciferase sequence was cloned downstream of the