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Analysis of the bone morphogenetic protein 6 gene promoter region in young beef cattle affected by enzootic bovine leukosis

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ABSTRACT. Enzootic bovine leukosis (EBL) is typically observed in cattle over 3 years old. However, some cases of EBL onset in young beef cattle have been reported in Japan. The mechanism for early EBL onset is unclear. In Japan, beef cattle are given large amounts of concentrated feed with low vitamin A. Bone morphogenetic proteins (BMPs) are regulators of cell proliferation, differentiation, and apoptosis, and thought to represent one of the key players in tumor malignancy. The purpose of this study was to evaluate the differences in BMP-6 methylation status between EBL beef cattle under 3 years old and other cattle. We investigated the methylation status of the BMP-6 promoter region in 32 EBL beef cattle under 3 years old. We also compared the methylation status of EBL dairy cattle to that of healthy cattle. Median methylation rate of the BMP-6 promoter region in EBL beef cattle under 3 years old was 8.9%, which was significantly higher than that of other groups. Hypermethylation of the BMP-6 promoter region might contribute to early onset of EBL in beef cattle under 3 years old, and animal feeding management practices specific to beef cattle may affect the methylation status of the BMP-6 promoter region.

KEY WORDS: beef cattle, bone morphogenetic protein 6, CpG islands, early onset, enzootic bovine leucosis, methylation

Enzootic bovine leukosis (EBL) is one of the most common neoplasms in cattle and is caused by bovine leukemia virus (BLV) [38]. BLV-infected cattle usually remain clinically silent without symptoms, and less than 5% of the infected animals develop EBL [10]. Because BLV requires a long latency period, median incubation period of EBL were estimated to be 7 years and EBL is typically observed in cattle over 3 years old [10, 42]. However, early onset of EBL in cattle under 3 years old has also been reported [25, 26, 33, 34], and the incidence of EBL onset in young beef cattle has increased in Japan [39]. Several factors relating to the progression of EBL from an asymptomatic state have been reported [22, 24]. Tax mutations and integration site of the BLV provirus are thought to influence the early onset of EBL [17, 25]. Moreover, intrauterine infection of BLV has been reported [37] and the incubation period may begin in the fetal period. However, detailed mechanisms for this remain unclear.

Epigenetic modifications including DNA methylation can alter the transcription and translation of particular genes to increase or decrease their functional levels in mammals [3]. Gene silencing caused by epigenetic modification is an important mechanism of inhibition of tumor suppressor gene expression in various cancers [15, 44]. DNA methylation refers to the addition of methyl groups to CpG dinucleotides in DNA [18, 32] and usually occurs in CpG islands with a high density of CpG dinucleotides [7]. Hypermethylation-associated gene silencing of tumor suppression, cell adhesion, and apoptosis has been described in malignant lymphoma cases in dogs, cats, and humans [6, 8, 9, 11, 12, 29]. Therefore, changes in methylation patterns are thought to be important for lymphomagenesis.

Bone morphogenetic proteins (BMPs), belonging to the transforming growth factor-β superfamily, are important regulators of cell proliferation, differentiation, and apoptosis [4]. In B-cells, BMP-6 reduces proliferation and induces apoptosis [21]. Moreover, repression of BMP-6 by methylation were found in human lymphoma [6, 41]. Therefore, inactivation of BMP-6 is thought to be a key player of tumorigenesis and progression in human lymphoma [6, 41]. However, little is known about the role of BMP-6 with regard to EBL onset.

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Accordingly, we hypothesized that repression of BMP-6 by methylation contributes to the onset of EBL in cattle. Moreover, we focused on methylation status of young beef cattle because methylation status was influenced by diet [36]. In the present study, we first compared the methylation status of the BMP-6 gene promoter region in EBL cattle to that of healthy cattle. Next, the methylation status of the BMP-6 gene promoter region in EBL beef cattle under 3 years old was compared with those of EBL dairy cattle and healthy cattle. Finally, we analyzed the association between methylation rates of the BMP-6 gene promoter region and BMP-6 gene expression.

MATERIALS AND METHODS

Samples and preparation of DNA

This study was approved by Obihiro University of Agriculture and Veterinary Medicine Committee for Experiments Using Animals (Approval number: 19-144) based on the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions. Samples from 117 cattle were provided by local veterinarians and meat hygiene inspection centers in Iwate and Hokkaido prefectures, Japan (Supplementary Tables 1–6). Samples comprised peripheral blood, neoplastic lymph nodes (mediastinal, superficial, mesenteric, subiliac, medial, or iliac lymph nodes) and tumor tissue (spleen, heart, lung, abomasum, liver, kidney, or uterus) from 67 EBL cattle (32 EBL beef cattle under 3 years old, 10 EBL dairy cattle under 3 years old, and 25 EBL dairy cattle over 3 years old). Peripheral blood and normal mesenteric lymph nodes from 50 healthy cattle (20 healthy beef cattle under 3 years old, 15 healthy dairy cattle under 3 years old, and 15 healthy dairy cattle over 3 years old) were also used. Beef cattle over 3 years old were not included in this study because most are slaughtered by 3 years old in Japan. The included breeds were Holstein-Frisian (HF, n=7), Japanese Black (JB, n=11) and crossbreeds of HF and JB (F1, n=14) in EBL beef cattle under 3 years old, and HF (n=9), JB (n=5), and F1 (n=6) in healthy beef cattle. All dairy cattle were HF. Production stages of 30 EBL beef cattle and 20 healthy beef cattle were fatting stage, and those of 2 EBL beef cattle were growing stage. Genomic DNA was extracted from each sample using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and stored at −30°C until analysis. EBL cattle were definitively diagnosed by macroscopic and/or histopathological examination at the Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan, and monoclonal integration of the BLV provirus was confirmed using inverse polymerase chain reaction (PCR) [30]. BLV infection in healthy cattle was confirmed by nested PCR for BLV 5′ LTR [26].

Bisulfite sequencing semi-nested PCR

Sodium bisulfite modification of isolated genomic DNA (1 µg) was performed using the MethylEasy Xceed Rapid DNA Bisulphite Modification Kit according to the manufacturer's instructions (Takara Bio, Kusatsu, Japan). The bisulfite treatment of DNA converts unmethylated CpG sites to UpG without modifying methlylated sites. Semi-nested PCR on bisulfite-treated DNA samples for CpG islands in the BMP-6 gene promoter region was performed with the following primers: F1/R2 (F1: 5′-AGGGGTAAGGGGAAATTT-3′, R2: 5′-CCTCCCCATTAACAAC-3′) for outer PCR and F1/R1 (R1: 5′-TCCCTCCCATCCCTTCTA-3′) for inner PCR, which yields a PCR product of 485 base pairs (bp). These primers were designed based on the sequence of CpG islands located in the BMP-6 gene promoter region. TaKaRa EpiTaq™ HS (for bisulfite-treated DNA) (Takara Bio) was used for the PCR. The first amplification program was carried out as follows: 40 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The second PCR was performed with the same protocol, except that the annealing temperature was set at 53°C. The PCR products from each sample were electrophoresed in 2% agarose gel and purified from the gels using QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were cloned into the pCRTM4-TOPO vector (Invitrogen, Carlsbad, CA, USA), and transformed into One Shot® TOP10 Chemically Competent *Escherichia coli*. Plasmid DNA from the isolated bacterial clones containing the insert was purified using the NucleoSpin Plasmid EasyPure (Takara Bio). Plasmid DNA samples from 10 independent clones for each sample were sequenced with the M13R primer. The DNA sequence was determined with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Norwalk, CT, USA) and ABI PRISM 3500 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. Fifty-six CpG sites of 10 clones in each sample were analyzed. Therefore, methylation rates were determined by the rate of methylated cytosines in 560 CpG sites.

Reverse transcription-PCR (RT-PCR) of the BMP-6 gene transcript

Neoplastic lymph nodes from 17 EBL cattle were collected in RNAlaterTM Stabilization Solution (Invitrogen), peripheral blood was collected from 10 healthy cattle in PAXgeneTM tubes (PreAnalytiX®, Hornbrechtikon, Switzerland), and both samples were collected from 3 EBL cattle in same methods. These samples were incubated overnight at room temperature and then at −80°C until analysis. Total RNA was extracted from neoplastic lymph nodes using the RNeasy Mini Kit (QIAGEN) and from peripheral blood using the PAXgeneTM Blood RNA Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using 2 µg total RNA and the SuperScriptTM III First-Strand Synthesis System (Invitrogen). PCR for messenger RNA (mRNA) of BMP-6 gene was performed using GoTaq Master Mix (Promega, Madison, WI, USA) with the following primer set: 5′-AGGACAGCGCCTTTCTCAAC-3′ and 5′-TTGTAGATTCGGAACTCTGCAGCC-3′, yielding a PCR product of 167 bp. These primers were designed based on the sequence of BMP-6 gene exons 1 and 2. The amplification program was carried out as follows: initial incubation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were electrophoresed in 2% agarose gel. RPS15A was used as a control for cDNA integrity and quality [14].

Statistical analyses

The Mann-Whitney *U* test was used to compare methylation rates of the BMP-6 gene promoter region between EBL cattle and healthy cattle, and between BLV-positive healthy cattle and BLV-negative healthy cattle. Methylation rates of the BMP-6 gene promoter region were statistically analyzed by using the Steel-Dwass and Kruskal-Wallis tests in 6 groups (EBL beef cattle under 3 years old, EBL dairy cattle under 3 years old, EBL dairy cattle over 3 years old, healthy beef cattle under 3 years old, healthy dairy cattle under 3 years old, and healthy dairy cattle over 3 years old). Moreover, median methylation rates of the BMP-6 gene promoter region in BLV-positive healthy cattle under 3 years old, BLV-positive healthy cattle over 3 years old, BLV-negative healthy cattle under 3 years old and BLV-negative healthy cattle over 3 years old were statistically analyzed by using Kruskal-Wallis tests. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed with EZR, a graphical user interface for R (The R Foundation for Statistical Computing, version 1.40) [20].

RESULTS

Methylation status of the BMP-6 gene promoter region in EBL and healthy cattle

Figure 1 shows the rates of hypermethylation in the BMP-6 gene promoter region in EBL cattle but do not represent the methylation status of the EBL group. We found that 498 out of 560 CpG sites were methylated (88.9% methylation rate). Methylation rates of the BMP-6 gene promoter region in EBL and healthy cattle are summarized in Fig. 2. Median methylation rates of the BMP-6 gene promoter region in EBL and healthy cattle were 5.4% (0.0–88.9%) and 1.8% (0.0–14.8%), respectively. Methylation rates of the BMP-6 gene promoter region in EBL cattle were significantly higher than those in healthy cattle (*P*<0.01 by Mann-Whitney *U* test).

Comparison of methylation rates of the BMP-6 gene promoter region between EBL beef cattle under 3 years old and other cattle

Median methylation rates of the BMP-6 gene promoter region in EBL beef cattle under 3 years old, EBL dairy cattle under 3 years old, EBL dairy cattle over 3 years old, healthy beef cattle under 3 years old, healthy dairy cattle under 3 years old, and healthy dairy cattle over 3 years old were 8.9% (2.3–88.9%), 3.2% (0.0–9.5%), 2.5% (0.0–46.8%), 2.7% (0.0–9.5%), 0.7% (0.0–3.1%), and 2.1% (0.0–14.8%), respectively (Fig. 3). Kruskal-Wallis test indicated significant difference of median methylation rates within the 6 groups (*P*<0.01), and median methylation rates of the BMP-6 gene promoter region in EBL beef cattle under 3 years old were significantly higher than those in the other 5 groups (*P*<0.05 by Steel-Dwass test) (Fig. 3). Moreover, methylation rates of the BMP-6 gene promoter region in EBL dairy cattle under 3 years old, EBL dairy cattle over 3 years old, and healthy beef cattle under 3 years old were significantly higher than those in healthy dairy cattle under 3 years old (*P*<0.05 by Steel-Dwass test) (Fig. 3).

Methylation rates of the BMP-6 gene promoter region in healthy cattle with and without BLV infection

BLV infection was detected in 25 of 50 healthy cattle. Median methylation rates of the BMP-6 gene promoter region were 1.8% (0.0–9.5%) in BLV-positive healthy cattle and 1.5% (0.0–14.8%) in BLV-negative healthy cattle, showing no significant difference (*P*=0.362 by Mann-Whitney U test). Moreover, median methylation rates of the BMP-6 gene promoter region in BLV-positive healthy cattle under 3 years old, BLV-positive healthy cattle over 3 years old, BLV-negative healthy cattle under 3 years old and BLV-negative healthy cattle over 3 years old were 1.8% (0.0–9.5%), 0.9% (0.0–5.4%), 1.4% (0.0–6.4%) and 2.1% (0.0–14.8%), respectively, suggesting no significant difference within those 4 groups (*P*=0.845 by Kruskal-Wallis test).

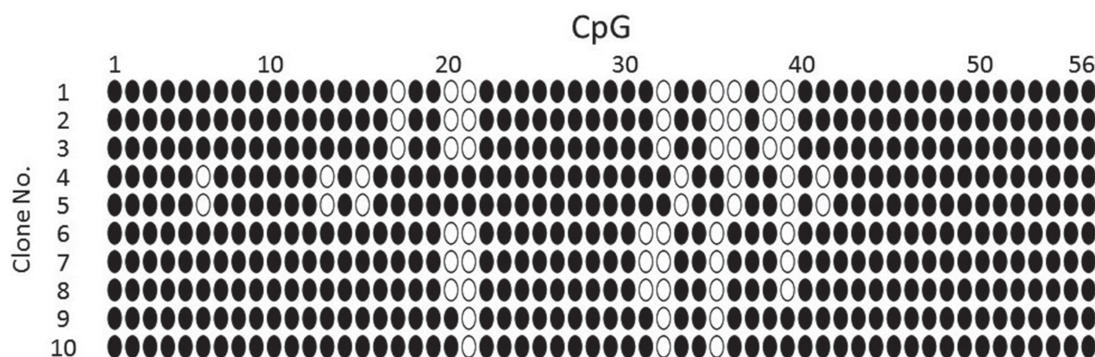

Fig. 1. Representative results in determining the bone morphogenetic protein 6 gene promoter hypermethylation in enzootic bovine leukosis (EBL) cattle but do not represent the methylation status of the EBL group. Ten clones were sequenced to obtain a representative sampling of methylation status. Fifty-six CpG sites in each sample were analyzed and numbered from left to right. In this case, 498 of 560 CpG sites were methylated (methylation rate, 88.9%). Methylated and unmethylated sites are shown by closed and open circles, respectively.

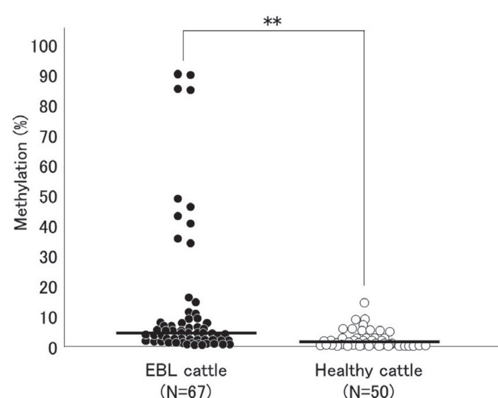

Fig. 2. Bone morphogenetic protein 6 gene promoter region methylation rates in enzootic bovine leukosis (EBL) cattle (black circles, n=67) and healthy cattle (white circles, n=50). ** indicates a significant difference (*P*<0.01 by Mann-Whitney *U* test). Bars represent the median.

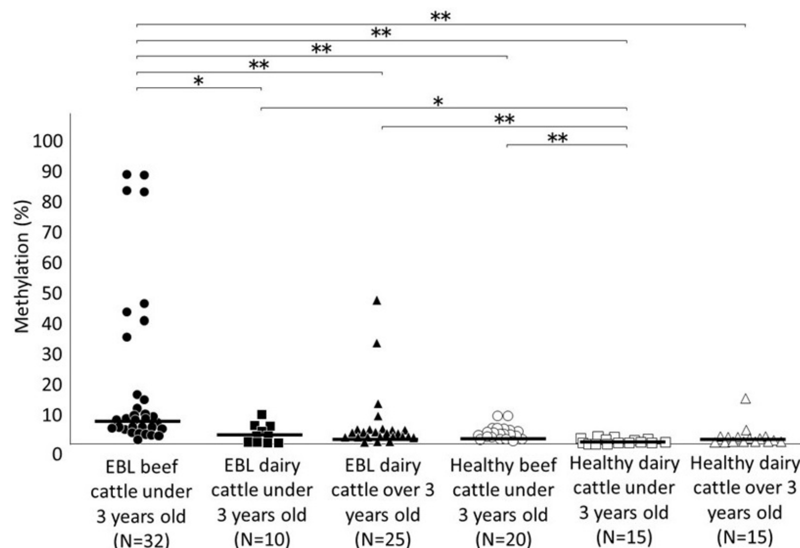

Fig. 3. Bone morphogenetic protein 6 gene promoter region methylation rates in enzootic bovine leukosis (EBL) beef cattle under 3 years old (black circles, n=32), EBL dairy cattle under 3 years old (black squares, n=10), EBL dairy cattle over 3 years old (black triangles, n=25), healthy beef cattle under 3 years old (white circles, n=20), healthy dairy cattle under 3 years old (white squares, n=15), and healthy dairy cattle over 3 years old (white triangles, n=15). * and ** indicate a significant difference (*: *P*<0.05, **: *P*<0.01 by Steel-Dwass test). Bars represent the median.

Association between methylation rates of the BMP-6 gene promoter region and transcriptional gene silencing

Results of RT-PCR of the BMP-6 gene of 20 EBL and 10 healthy cattle are shown in Fig. 4 and Table 1. The range of methylation rates of the BMP-6 gene promoter region in those cattle was 0.0–88.6%. Expression of BMP-6 gene mRNA was found in samples with methylation rates under 9.5%, but not in samples with those over 33.4% (Table 1). All samples expressed the housekeeping gene of RPS15A mRNA transcripts (Fig. 4 and Table 1).

DISCUSSION

EBL cattle show monoclonal expansion of B cells with identical BLV proviral integration sites, whereas other BLV-infected cattle show diverse integration sites [28]. Thus, EBL is strongly suspected by detection of BLV provirus monoclonal integration in cattle with B-cell lymphoma [34]. EBL is typically observed in cattle over 3 years old [10], and detection of monoclonal integration of BLV provirus was a useful method for diagnosing EBL in cattle with difficult diagnosis, such as young cattle [34]. Accordingly, in the present study, we examined monoclonal BLV proviral integration by inverse PCR in order to diagnose EBL. However, the sensitivity and specificity of inverse PCR as diagnostic method of EBL are not known. Further investigation is required to clear the usefulness of inverse-PCR method for diagnosing EBL.

BMP-6 is involved in fine tuning the balance between proliferation, apoptosis, and differentiation in human B progenitor cells [21], and is thought to play a role in the genesis and progression of many tumors. For instance, methylation rates of the BMP-6 gene promoter region were increased in accordance with disease progression in adult T-cell leukemia [41], and concurrent repression of BMPs including BMP-6 by methylation was important in the onset of lung cancer [23]. In the present study, methylation rates of the BMP-6 gene promoter region in EBL cattle were significantly higher than those in healthy cattle, and the methylation-dependent loss of BMP-6 gene expression at the mRNA level was observed. This suggests that methylation rate exceeding 33% of the BMP-6 gene promoter region might cause BMP-6 gene transcriptional repression and contribute to EBL onset.

Factors known to be associated with methylation status include aging, diet, chronic inflammation, and viral infection [2, 16, 19, 31, 36]. In the present study, the methylation rates of BMP-6 gene promoter region in EBL beef cattle under 3 years old were significantly higher than those in EBL dairy cattle under 3 years old. Moreover, methylation rates of the BMP-6 gene promoter region in healthy beef cattle under 3 years old were also significantly higher than those in healthy dairy cattle under 3 years old. In Japan, dairy cattle are fed mainly on forage grasses, whereas almost all of the beef cattle in fatting stage are given a lot concentrated feed that contain very little V.A [27, 35]. Serum concentrations of V.A and total cholesterol of beef cattle in fatting stage were approximately 30 IU/dl and 150 mg/dl, respectively [1]. Decreased V.A intake and hyperlipidemia cause and promote inflammatory diseases [5, 13], and beef cattle often develop V.A deficiency and inflammatory diseases [27]. Therefore, feed management practices for beef cattle might contribute to methylation of the BMP-6 gene promoter region, potentially leading to early EBL onset. However, it remains unclear which factors

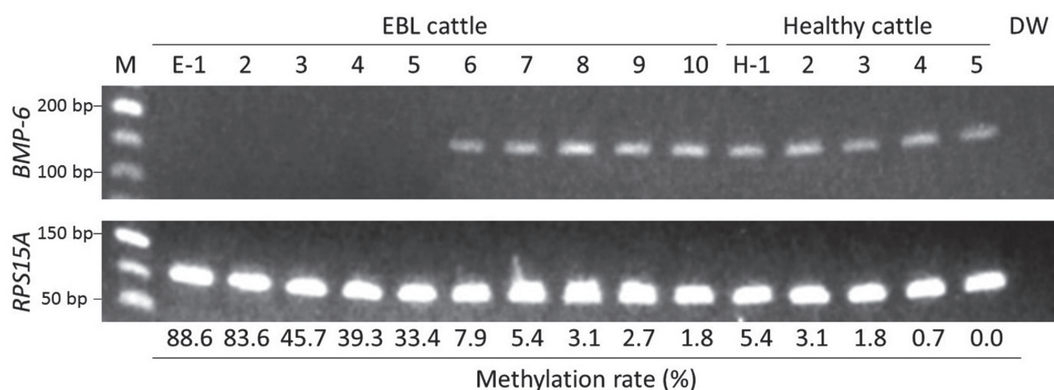

Fig. 4. Representative results of reverse transcription-polymerase chain reaction of bone morphogenetic protein 6 (BMP-6) gene (167 bp) for lymph nodes and peripheral blood samples in enzootic bovine leukosis (EBL) and healthy cattle. The range of methylation rates of the BMP-6 gene promoter region in those cattle was 0.0–88.6%. RPS15A was used as a control for cDNA integrity and quality. M and DW denote molecular weight marker and distilled water, respectively.

related to beef cattle affected the methylation status of BMP-6 promoter region. Further investigation is required to clarify the factors that caused hypermethylation of the BMP-6 gene promoter region. In 2 dairy EBL cattle over 3 years old, methylation levels of the BMP-6 gene promoter region were increased. The age of those cattle were 112 and 140 month-old (Supplementary Table 3). Although methylation status of BMP-6 promoter region in those cattle might be influenced by age, cause of hypermethylation was unclear. Methylation rates of the BMP-6 gene promoter region did not differ significantly between BLV-positive healthy cattle and BLV-negative healthy cattle, suggesting that BLV infection may not affect methylation rates of the BMP-6 gene promoter region in healthy cattle.

Although higher methylation rates of the BMP-6 gene promoter region were observed in EBL cattle compared to those in healthy cattle, median methylation rates of the BMP-6 gene promoter region in EBL cattle were comparable to those observed in cattle with BMP-6 gene expression. Low levels of random methylation within the CpG islands may trigger or promote hypermethylation of the surrounding CpG sites [40]. Moreover, some gastric cancer cell lines tend to have scattered methylation within CpG island, which leads to dense methylation [43]. Therefore, sparse methylation, even at levels that do not affect transcriptional activity, may increase the risk for hypermethylation and onset of EBL.

In the present study, samples were mainly lymph nodes and peripheral blood in EBL cattle and healthy cattle, respectively. Methylation statuses might be different between blood and lymph nodes. Additional studies on methylation status of BMP-6 promoter region in same tissue samples were needed to eliminate sample bias. Moreover, in the present study, multiple samples from same animals were able to collect in only few cattle. Further investigation is required to clear the characteristics of methylation status of BMP-6 promoter region in each tissue and peripheral blood.

In conclusion, the present study revealed that the BMP-6 gene promoter region hypermethylation was more common in EBL cattle than in healthy cattle. Moreover, methylation rates of the BMP-6 gene promoter region

Table 1. Bone morphogenetic protein 6 (BMP-6) promoter methylation status and BMP-6 expression in enzootic bovine leukosis (EBL) and healthy cattle

Cattle No.	Sample	Methylation rate (%)	Expression	
			BMP-6	*RPS15A*
EBL-1	Lymph node	88.6	-	+
EBL-2	Lymph node	83.6	-	+
EBL-3	Peripheral blood	42.9	-	+
	Lymph node	45.7	-	+
EBL-4	Lymph node	39.3	-	+
EBL-5	Lymph node	33.4	-	+
EBL-6	Lymph node	7.9	+	+
EBL-7	Peripheral blood	5.2	+	+
	Lymph node	5.4	+	+
EBL-8	Peripheral blood	3.8	+	+
	Lymph node	3.1	+	+
EBL-9	Lymph node	2.7	+	+
EBL-10	Lymph node	1.8	+	+
EBL-11	Lymph node	9.5	+	+
EBL-12	Lymph node	46.8	-	+
EBL-13	Lymph node	5.5	+	+
EBL-14	Lymph node	3.6	+	+
EBL-15	Lymph node	3.2	+	+
EBL-16	Lymph node	2.7	+	+
EBL-17	Lymph node	2.3	+	+
EBL-18	Lymph node	1.8	+	+
EBL-19	Lymph node	1.1	+	+
EBL-20	Lymph node	0.0	+	+
Healthy-1	Peripheral blood	5.4	+	+
Healthy-2	Peripheral blood	3.1	+	+
Healthy-3	Peripheral blood	1.8	+	+
Healthy-4	Peripheral blood	0.7	+	+
Healthy-5	Peripheral blood	0.0	+	+
Healthy-6	Peripheral blood	5.5	+	+
Healthy-7	Peripheral blood	3.2	+	+
Healthy-8	Peripheral blood	2.1	+	+
Healthy-9	Peripheral blood	0.7	+	+
Healthy-10	Peripheral blood	0.0	+	+

+: Expression was detected by RT-PCR. -: Expression was not detected by RT-PCR.

in EBL beef cattle under 3 years old were significantly higher than those in EBL dairy cattle under 3 years old. This suggests that hypermethylation of the BMP-6 gene promoter region might represent a factor contributing to early EBL onset in beef cattle.

POTENTIAL CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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