NOTE *Clinical Pathology*

Evaluation of Gene Expression in Peripheral Blood Cells as a Potential Biomarker for Enzootic Bovine Leukosis

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ABSTRACT. Expression of six selective genes in peripheral blood cells was evaluated as diagnostic biomarkers for enzootic bovine leukosis (EBL) by using 10 EBL and 15 clinically healthy cattle. The clinically healthy cattle generally showed lower gene expression levels. Although wide variations of gene expression were found in some clinical cases of EBL, 4 and 5 among 10 EBL cattle showed higher expression of interleukin 2 receptor gene (*IL2R*) and *Wilms'* tumor gene (*WT1*), respectively. Expression of *IL2R* in peripheral blood cells in EBL cattle was statistically increased; however, the lower sensitivity and higher variation in the gene expressions among clinical cases of EBL would be problems as diagnostic biomarkers.

KEY WORDS: biomarkers, enzootic bovine leukosis, gene expression, qRT-PCR.

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Bovine leukosis/lymphsarcoma (BL) is one of the most common neoplastic diseases of cattle and has been classified into two types according to pathological, epizootiological and clinicopathological findings. These include sporadic bovine leukosis, which has no known cause and enzootic bovine leukosis (EBL), which is associated with the bovine leukemia virus (BLV) [5, 21]. The vast majority of animals with BLV remain persistently affected with no sign of infection and approximately 29% of cattle infected with BLV develop persistent lymphocytosis (PL), while fewer than 5% of animals affected by BLV develop lymphsarcoma [11]. Clinical signs of cattle affected by EBL are general malaise, decreased milk production, enlarged superficial lymph nodes, anorexia, abomasal ulceration, cardiac lesion and exophthalmos [3, 22]. Findings that lead to suspicions of EBL include lymphocytosis, enlargement of peripheral lymph nodes and the presence of neoplastic lymphocytes in peripheral blood [12]. In general, fine needle aspiration (FNA) cytology of primary neoplasms or neoplastic lymph nodes can lead to a definitive diagnosis of EBL, but the sensitivity and specificity of FNA are not confidence [30]. Furthermore, diagnosis can be difficult for EBL which lacks lymphocytosis and enlargement of lymph nodes [12]. Thus, more reliable biomarkers are recently required to diagnose EBL. With respect to bovine leukemia, higher activity of serum lactate dehydrogenase (LDH) and LDH isozymes have been used as biomarkers to diagnose lymphsarcoma, even though LDH is not necessarily more specific for EBL and is expressed in other diseases as well [15]. In addition, higher serum thymidine kinase activity has recently demonstrated potential as a biomarker for clinical diagnosis of EBL, but this requires a radioimmunoassay test [23].

Genomic biomarkers are increasing in popularity for diagnosis of certain diseases within the field of human medicine. For example, the Wilms' tumor 1 (*WT1*) gene is used as a biomarker due to its high expression levels in hematological malignancies and various cancers and low levels in normal tissues [20, 29]. Additionally, high B-cell lymphoma/leukemia protein 2 (*BCL2*) activities have been found in mature peripheral B-cell neoplasms, such as those in B-cell chronic lymphocytic leukemia [1, 24]. Although veterinary medicine would benefit greatly from similar methods and markers for clinical diagnosis of EBL, there has been little information available. Thus, the present study evaluated mRNA expression levels of several target genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Specifically, we analyzed interleukin 2 receptor (*IL2R*), *WT1*, thymidine kinase 1 (*TK1*), cytochrome P450 family 1-subfamily B- polypeptide 1 (*CYP1B1*), *BCL2* and phosphodiesterase isoform 7B (*PDE7B*) to diagnose clinical cases of EBL in cattle.

A total of 25 cattle, including 15 clinically healthy and 10 EBL cattle, were used in this study. Profiles of these 25 cattle are summarized in Table 1. All the 15 healthy cattle were kept in a farm and monitored annually for BLV infection and complete blood counts. These healthy cattle were divided into the following three groups by the hematological examination and BLV status; (1) five clinically healthy cattle with neither BLV infection nor PL (BLV^-PL^-), (2) five clinically healthy cattle with BLV infection, but no PL (BLV^+

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Table 1. Profiles of cattle used in this study

No.	Breed	Age (Month)	Total lymphocytes (/μl)	Atypical lymphocytes (/μl)	Fold changes of gene expression					
					IL2R	*WT1*	*TK1*	*CYP1B1*	*BCL2*	*PED7B*
I. Clinically healthy cattle without BLV nor lymphocytosis (BLV−PL−)										
1	HF	64	2,580	NE	0.703	0.379	0.735	2.151	1.121	0.972
2	HF	63	2,250	NE	1.216	0.930	0.955	0.324	1.327	1.030
3	HF	54	3,066	NE	1.276	1.017	0.929	0.345	0.949	1.097
4	HF	54	1,932	NE	1.141	2.743	1.489	2.403	0.956	1.339
5	HF	42	3,555	NE	0.803	1.016	1.030	1.735	0.740	0.680
Median		54	2,580	-	1.141	1.016	0.955	1.735	0.956	1.030
II. Clinically healthy cattle with BLV, but without lymphocytosis (BLV+PL−)										
6	HF	63	5,478	NE	0.650	1.134	1.349	4.584	2.178	0.298
7	HF	60	2,232	NE	0.886	1.036	0.588	1.193	1.005	1.949
8	HF	55	4,550	NE	1.828	1.433	1.602	0.278	0.773	2.454
9	HF	52	4,171	NE	0.699	1.237	0.834	0.148	0.971	1.673
10	HF	40	5,142	NE	0.331	0.763	0.433	0.176	0.608	0.600
Median		55	4,550	-	0.699	1.134	0.834	0.278	0.971	1.673
III. Clinically healthy cattle with BLV and lymphocytosis (BLV+ PL+)										
11	HF	68	12,844	NE	0.268	0.186	0.697	2.761	0.618	0.986
12	HF	65	7,772	NE	2.693	5.390	2.131	1.826	1.371	2.885
13	HF	53	11,248	NE	0.601	NT	1.959	0.454	1.852	1.359
14	HF	52	12,874	NE	0.413	NT	0.966	0.157	0.724	0.492
15	HF	45	11,078	NE	0.269	NT	0.717	0.033	0.881	0.324
Median		53	11,248	-	0.413	2.788	0.966	0.454	0.881	0.986
IV. EBL without lymphocytosis (EBL+ BLV+ PL−)										
16	HF	115	4,662	0	22.072	0.063	0.201	0.061	1.521	2.689
17	HF	83	2,948	0	21.110	0.069	0.457	0.008	0.798	1.304
18	HF	71	2,640	0	1.424	0.856	0.270	0.434	0.433	0.662
19	HF	71	4,905	1,635	8.293	2.692	1.838	1.122	1.345	3.808
Median		77	3,805	0	14.702	0.463	0.364	0.248	1.072	1.997
V. EBL with lymphocytosis (EBL+BLV+PL+)										
20	HF	85	12,950	2,100	8.641	0.198	0.684	0.173	0.435	1.612
21	HF	73	39,690	38,808	0.085	0.434	2.015	2.055	1.718	0.397
22	HF	36	441,196	247,610	0.038	5.100	1.231	2.536	7.859	0.399
23	JB	36	303,513	306,642	0.047	10.162	2.190	130.710	0.628	0.767
24	HF	30	21,248	1,660	1.523	2.305	0.793	0.173	3.018	6.379
25	HF	18	221,664	219,355	0.091	6.852	7.458	30.197	0.127	0.091
Median		36	130,677	129,082	0.088	3.703	1.623	2.296	1.173	0.583

NE: Not evaluated, NT: Not tested, HF: Holsten-Friesian, JB: Japanese Black.

PL$^-$) and (3) five clinically healthy cattle with both BLV infection and PL, but no onset of EBL (BLV$^+$PL$^+$). Peripheral lymphocyte numbers of all the cattle examined in the present study were evaluated by Bendixen's key criteria [5]. BLV infection was evaluated by using both real-time PCR kit for BLV tax-gene (CycleavePCR®, Takara Biotechnology Co., Ltd., Otsu, Japan) and agar-gel immunodiffusion (Kitasato Institute Research Center for Biologicals, Kitamoto, Japan). The onset of EBL was not observed in these 15 cattle at least for 14 months since the time of sample collection on November, 2011. Each EBL cattle was kept at different farms, and samples were collected from April to September 2012. All the 10 EBL cattle showed lymphadenopathy and BLV positive. Cytology findings of fine needle aspiration samples of enlarged lymph nodes confirmed definitive diagnosis of EBL for these 10 cattle. The EBL cattle were divided into 2 groups by the numbers of peripheral lymphocytes: (4) four EBL cattle without lymphocytosis (EBL$^+$BLV$^+$PL$^-$) and (5) six EBL cattle with lymphocytosis (EBL$^+$BLV$^+$PL$^+$).

Table 2. Primers used in this study

Gene	Forward	Reverse
IL2R	cgccatgttcaaggtcttc	gttctgcgcatctgtgtgtt
WT1	ttctcgttcagaccagctca	gctgaagggcttctcacttg
TK1	ccaggttgcccagtacaagt	tctcgcagaactccacaatg
CYP1B1	tgttaaccagtggtccgtga	ttgaaattgcactggtgagc
BCL2	ctgacttctctcggcgctac	cggttcaggtactcggtcat
PDE7B	caggccatgcactgctac	ttggcaagtgagccagaa
ACTB	ctcttccagccttccttcct	gggcagtgatctctttctgc

A total of 2.5 m*l* of peripheral blood was collected in PAX-

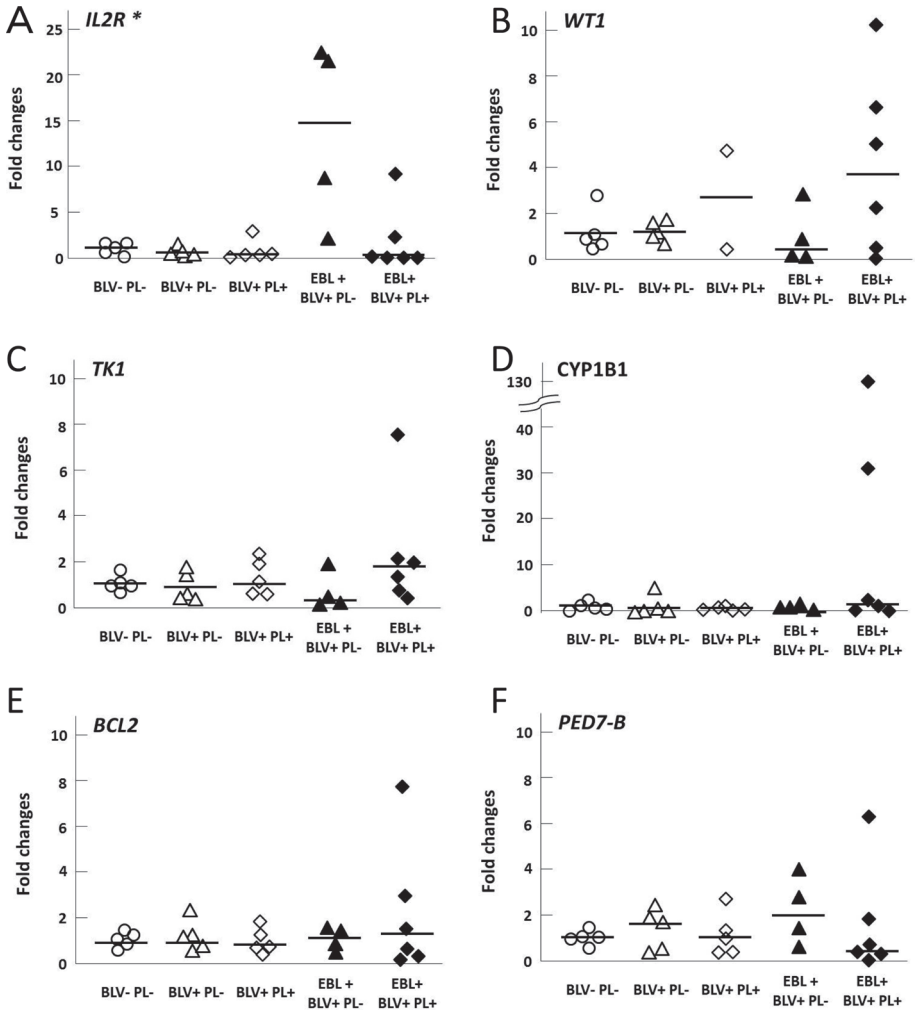

Fig. 1. Gene expression levels in peripheral blood of cattle from 5 different groups as measured by quantitative RT-PCR targeting the following genes: (A)*IL2R*: interleukin-2 receptor; (B)*WT1*: Wilms' tumor 1; (C)*TK1*: thymidine kinase 1; (D)*CYP1B1*: cytochrome P450, family1- subfamily B- polypeptide 1; (E)*BCL2*: B-cell leukemia/lymphoma protein 2; and (F)*PDE7B*: phosphodiesterase isoform 7B. BLV−PL −: clinically healthy cattle tested negative for bovine leukemia virus (BLV) and showed no persistent lymphocytosis; BLV+PL−: clinically healthy cattle tested positive for BLV and showed no PL; BLV+ PL+: clinically healthy cattle tested positive for BLV and PL; EBL+ BLV+ PL −: enzootic bovine leukosis cattle without lymphocytosis; EBL+ BLV+ PL+: enzootic bovine leukosis cattle with lymphocytosis. Bars represent median. *: indicates a significant difference at P=0.047 by Kruskal Wallis test.

gene™ Blood RNA tubes (PreAnalytiX®, Hornbrechtikon, Switzerland) from each animal, incubated for at least 1–2 hr at room temperature and then kept at −30°C until analysis. Total RNA was extracted from peripheral blood using the PAXgene™ Blood RNA Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Reverse transcription was carried out using 0.5 μg RNA for cDNA synthesis using a thermo script system (Applied Biosystems, Foster City, CA, U.S.A.). Following the manufacturer's protocol, qRT-PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems) using a StepOnePlus System (Applied Biosystems). Messenger RNA (mRNA) expressions of *IL2R*, *WT1*, *TK1*, *CYP1B1*, *BCL2* and *PDE7B* were evaluated using beta actin (*ACTB*) as an endogenous control. The primers used in this study are listed in Table 2. All primers were designed using Primer Express (Applied Biosystems). Relative differences in gene expression were calculated using cycle time (Ct) values that were first normalized to those of beta actin and then to a control Ct value. Data of each group were statistically analyzed by using Kruskal Wallis test. A P-value of less than 0.05 was considered statistically significant.

The results are summarized in Table 1 and Fig. 1. All 3 groups of the clinically healthy cattle generally showed

lower expression levels for each gene. Except for *WT1* in BLV^+PL^+ group, the median of fold changes of *IL2R, WT1, TK1, CYP1B1, BCL2* and *PED7B* expression in the clinically healthy cattle showed lower values from 0.278 to 1.753 (Table 1). The results suggested that BLV infection and following persistent lymphocytosis do not significantly affect these gene expression examined in this study.

A total of 4 among 10 EBL cattle (40%) (3 cattle in $EBL^+BLV^+PL^-$ group and 1 cattle in $EBL^+BLV^+PL^+$ group) showed higher (more than 8 fold changes) expression of *IL2R* relative to that observed in the clinically healthy cattle (Fig.1A), whereas 4 in $EBL^+BLV^+PL^-$ group showed lower fold change less than 1.0 (Table 1). Statistical analysis revealed that the significant difference of *IL2R* expression was found among five groups (P=0.047). Interestingly, the IL2R expression in $EBL^+BLV^+PL^+$ group did not differ from that observed in 3 groups of clinically healthy cattle (Fig. 1A). These results are consistent with a previous study that showed lower *IL2* expression levels in cattle infected with BLV and showed persistent lymphocytosis, compared to the uninfected control cattle with no lymphocytosis [2]. In a previous study, we also observed higher *IL2R* expression in a clinical case of EBL with normal lymphocyte counts [28]. *IL2R* is a heterotrimeric protein expressed on the surface of lymphocytes and the receptor for interleukin 2 and is thought to be directly or indirectly activated by retro virus viral products [18, 31]. *IL2R* gene overexpression has been reported in human leukemia cases [10]. These findings suggest that monitoring expression of *IL2R* in peripheral blood may be a feasible biomarker for EBL, but some EBL cattle showed lower expression. Although the exact reason why some EBL cases showed lower *IL2R* expression is unknown, the variety of clinical courses and stages of EBL in patients might affect the results. Further studies are required to clarify it.

A total of 5 among 10 EBL cattle (50%) (4 cattle in $EBL^+BLV^+PL^-$ group and 1 cattle in $EBL^+BLV^+PL^+$ group) showed higher *WT1* expression levels. The median value of *WT1* in the $EBL^+BLV^+PL^+$ group showed higher than that of other 4 groups (Fig. 1B), although there were no significant differences among 5 groups. *WT1* encodes a zinc finger transcription factor required for cell growth and differentiation in several organs [6, 26]. This gene is highly expressed in most cases of acute myeloid leukemia and in almost all types of solid tumors in humans [13, 19]. It is possible that *WT1* gene in peripheral blood cells is more expressed in acute stage of the diseases. Lower sensitivity and wide variation of *WT1* gene expression in EBL were thought to be problem as a biomarker for EBL. As some of the clinically healthy cattle showed higher expression, it might contribute to the no significant difference between each group.

Small numbers of the EBL cattle showed extremely higher fold change values compared with 3 groups of clinically healthy cattle (Fig. 1C, D, E and F) in *TK1, CYP1B1, BCL2* and *PED7B*. However, the rest of EBL cattle showed very lower levels (less than 1.0) of gene expression similar to those of 3 groups of clinically healthy cattle, and the median values of each gene in EBL cattle also showed lower as non-EBL groups. Although the potential usefulness of gene expression of *TK1, CYP1B1, BCL2* and *PED7B* as biomarkers of malignant hematopoietic cell tumors in human [4, 7–9, 14, 16, 17, 25, 27, 28, 32], the present data suggest that these genes are poor biomarkers for EBL onset, because of their lower sensitivity. Wide variation of stage and distribution of the tumor might contribute to the results. It is notable that some of the EBL cattle showed very higher expression of *TK1, CYP1B1, BCL2* and *PED7B.*

The present study evaluated some specific genes related to tumor biomarkers of human for potential biomarkers for EBL. Some EBL cattle showed higher mRNA expression levels in some genes compared with the clinically healthy cattle. Although expression of *IL2R* and *WT1* in peripheral blood cells could be used as feasible biomarkers for clinical diagnosis of EBL, the lower sensitivity and higher variation in the gene expressions among clinical cases of EBL would be problems as diagnositic biomarkers. Future studies are required to clarify the relationship of the variety of clinical courses and stages of EBL with the gene expression to confirm the clinical utility of using gene expression levels for diagnoses.

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