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

Mohammad Monir TAWFEEQ1, 2) Noriyuki HORIUCHI3), Yoshiyasu KOBAYASHI3), Hidefumi FURUOKA3) and Hisashi INOKUMA1)*

1)Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, 080–8555, Japan
2)Faculty of Veterinary Science, Kabul University, Jamal Mina Kabul, Afghanistan
3)Department of Basic Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, 080–8555, Japan

(Received 1 March 2013/Accepted 2 April 2013/Published online in J-STAGE 16 April 2013)

**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.

Bovine leukemia/lymphosarcoma (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 leukemia, which has no known cause and enzootic bovine leukemia (EBL), which is associated with the bovine leukemia virus (BLV) [5, 21]. The vast majority of animals with BLV remain persistently infected without sign of infection and approximately 29% of cattle infected with BLV develop persistent lymphocytosis (PL), while fewer than 5% of animals infected by BLV develop lymphosarcoma [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 lymphosarcoma, 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−), (2) five clinically healthy cattle with BLV infection, but no PL (BLV+)...
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+).

A total of 2.5 ml of peripheral blood was collected in PAX-
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, family 1, 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.
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− 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 heterotrimetric 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 diagnostic 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.

ACKNOWLEDGMENTS. We thank the veterinarians of Tokachi Agricultural Mutual Aid Association for sampling. This work was supported in part by the Ito Foundation.

REFERENCES


