NOTE *Internal Medicine*

Detection of circulating tumor cells using GeneScan analysis for antigen receptor gene rearrangements in canine lymphoma patients

Saaya HIYOSHI-KANEMOTO[1)], Yuko GOTO-KOSHINO[1)], Kenjiro FUKUSHIMA[1)], Masashi TAKAHASHI[1)], Hideyuki KANEMOTO[1)], Kazuyuki UCHIDA[2)], Yasuhito FUJINO[1)], Koichi OHNO[1)] and Hajime TSUJIMOTO[1)]*

[1)]*Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan*
[2)]*Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan*

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ABSTRACT. The presence of circulating tumor cells (CTCs) serves as a prognostic marker and indicator of disease relapse, as well as a means of evaluating treatment efficacy in human and canine lymphoma patients. As an extension of our previous study for the construction of clinically useful GeneScan system, we utilized the GeneScan system for detecting CTCs in canine lymphoma patients. Samples from the primary lesion and peripheral blood mononuclear cells (PBMCs) were obtained from 32 dogs with lymphoma at initial diagnosis. All samples were subjected to polymerase chain reaction (PCR) for antigen receptor gene rearrangements (PARR) followed by GeneScan analysis. Common clonal rearrangements with identical amplified fragments were detected in both the primary lesion and PBMCs in 19 of the 32 dogs (59.4%). However, the detection rate of CTCs varied among the anatomical classification of lymphoma studied. GeneScan analysis following PARR would facilitate studies on determining the clinical significance of CTCs in canine lymphoma patients.
KEY WORDS: antigen receptor gene rearrangements, canine, circulating tumor cells, GeneScan, lymphoma

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Lymphoma is the most common hematopoietic neoplasm in dogs, occurring in 13−107 of every 100,000 dogs per year [4, 5, 15]. Multidrug chemotherapy based on the CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) protocol has been a standardized regimen for the treatment of typical high-grade lymphomas in dogs, showing complete remission (CR) rates of more than 80% [3, 7, 10]. However, most dogs experience relapse and eventually succumb to disease progression [7].

Circulating tumor cells (CTCs) are defined as tumor cells in the peripheral blood derived from primary or secondary tumor lesions. CTCs that escape anti-cancer therapy are considered mediators of disease relapse [13, 16]. Assessment of CTCs using molecular biology techniques after initiating chemotherapy is useful for predicting disease prognosis and relapse, as well as monitoring treatment efficacy in canine lymphoma [17–19, 23].

Detection of neoplastic cells by microscopic evaluation of peripheral blood is a routine laboratory examination. However, it is not objective and specific, allowing misdiagnosis, particularly when the peripheral blood contains only a small number of malignant cells. To overcome this limitation, several molecular techniques for detecting CTCs have been developed. Polymerase chain reaction (PCR) for antigen receptor gene rearrangements (PARR) was shown to be able to accurately detect CTCs [11, 14, 21]. This method detects clonal expansion of lymphoid cells by amplifying the rearranged *T-cell receptor gamma-chain* (*TCRγ*) gene or *immunoglobulin heavy-chain* (*IgH*) gene. The conventional PARR method is generally conducted by amplifying the complementary determining region 3 (CDR3) of antigen receptor genes with consensus primers annealing to variable (V) and joining (J) segments, followed by separation of DNA fragments using polyacryl amide gel electrophoresis (PAGE).

We developed a quantitative assessment method for detecting CTCs in canine lymphoma by real-time PCR [24]. This method is highly accurate, since the antigen receptor genes are amplified with individually prepared tumor-specific primers in each case after sequencing the rearranged allele.

Recently, GeneScan analysis was further introduced to examine the clonal expansion of lymphoid cells in dogs with lymphoproliferative disorders [8, 12]. In GeneScan analysis, the nucleotide length of the amplified fragments can be accurately defined based on size standards, thus enabling strict confirmation of a clonally rearranged band in the same individual.

Comparison among above methods for detecting CTCs is as follows: PAGE and GeneScan are not tumor specific and not sensitive as real-time PCR. Real-time PCR is labor intensive compared to PAGE and GeneScan, because it requires nucleotide sequencing analysis for each patient. However, it is quantitative and sensitive enough to detect minimal residual disease (MRD) even in clinical remission. Although

*CORRESPONDENCE TO: TSUJIMOTO, H., Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan. e-mail: atsuji@mail.ecc.u-tokyo.ac.jp

GeneScan has detection limit similar to that of PAGE, it enables us to define the size of PCR product, thereby being more tumor-specific compared to PAGE.

As an extension of our previous study for the construction of clinically useful GeneScan system, we utilized the GeneScan system for detecting CTCs in canine lymphoma patients. In a current study, we hypothesized that consensus primers could indicate the existence of CTCs, and we compared the PCR products between the primary tumor and peripheral blood using GeneScan analysis.

We investigated dogs diagnosed with lymphoma, which were referred to the Veterinary Medical Center of the University of Tokyo (VMC-UTokyo) from June 2011 to November 2013. Samples of both the primary lesion and peripheral blood mononuclear cells (PBMCs) were obtained at initial diagnosis. Dogs with concurrent inflammatory diseases, such as infection, parasitism and autoimmune diseases, at the time of diagnosis were excluded.

Consequently, a total of 32 dogs with lymphoma were included and classified as follows: 17 dogs with high-grade multicentric lymphoma (Cases 1–17), 12 dogs with gastrointestinal (GI) lymphoma (Cases 18–29) and three dogs with cutaneous lymphoma (Cases 30–32).

The dogs consisted of 14 males (9 neutered) and 18 females (9 neutered). The median age at the time of diagnosis was 9.9 years (range: 2.8–15.1 years). The dog breeds were as follows: Pembroke Welsh Corgi (n=5), Miniature Dachshund, Pug (4 each), French Bulldog, Miniature Schnauzer (3 each), Shih Tzu, Maltese, Shiba (2 each) and others (n=7).

Detailed information of sample acquisition and diagnosis are shown in "Supplementary file 1".

Genomic DNA was extracted from both primary lesions and PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Rearrangements of *IgH* and *TCRγ* genes for all specimens were assessed by amplifying CDR3 and analyzed by the GeneScan analytical system to determine the clonality of canine lymphoid cells, as previously described [9]. Detailed information of PARR followed by GeneScan analysis is shown in "Supplementary file 2".

Detection of the common peak(s), indicating the clonal rearrangement of antigen receptor genes between lesional and PBMC samples, was evaluated to indicate the existence of CTCs in each canine lymphoma patient.

Amplified PCR products were cloned into the pGEM-T Easy Vector using the TA cloning system (Promega Corporation, Madison, WI, U.S.A.) according to the manufacturer's instructions. Nucleotide sequencing was performed on the prepared plasmid using a BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and Applied Biosystems 3130xl genetic analyzer (Applied Biosystems).

Cases with clonal rearrangements of antigen receptor genes, confirmed in the primary lesion, were included in this study.

In 19 of the 32 canine lymphoma patients, clonal PCR products of identical antigen receptor gene nucleotide lengths were found in both the primary lesion and PBMCs. Representative capillary electropherograms of patients with each lymphoma subtype are shown in Fig. 1. CTC detection rates varied among the lymphoma subtypes: 13 of 17 dogs (76%) with high-grade multicentric lymphoma, four of 12 dogs (33%) with GI lymphoma and two of three dogs (67%) with cutaneous lymphoma. The sequences of PCR products common to the lesional and PBMC samples were analyzed in order to confirm CTC detection in two representative cases: one dog with high-grade multicentric lymphoma (Case 7) and one dog with GI lymphoma (Case 29) (Fig. 2). The PBMC sequences were identical to that of the primary lesions in seven of seven clones (100%) in Case 7 (Fig. 2a) and five of 12 clones (42%) in Case 29 (Fig. 2b), indicating the presence of CTCs.

Clonal rearrangements were detected by PARR in the

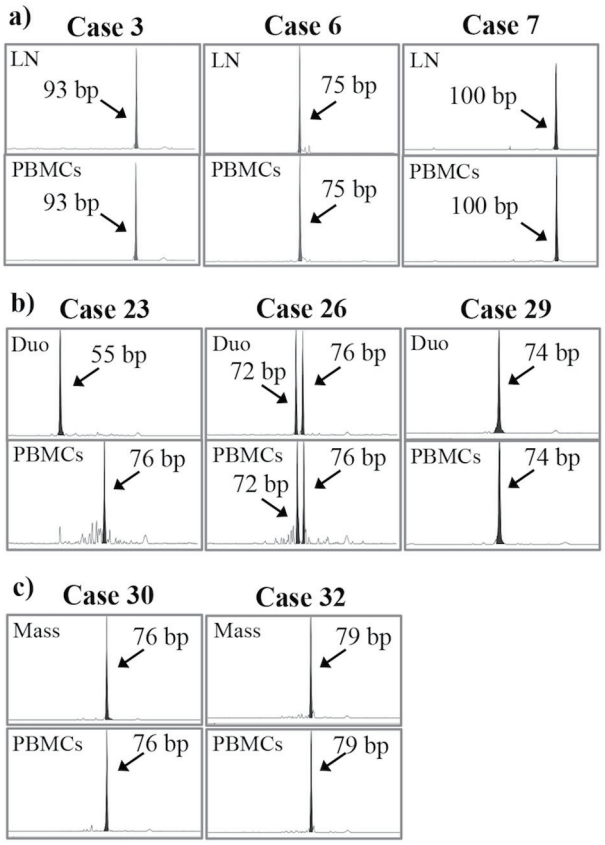

Fig. 1. Representative electropherograms analyzed by GeneScan in patients with each lymphoma subtype. The top and the bottom panels in each case show the electropherograms of the lesional sample and peripheral blood mononuclear cells (PBMCs), respectively. (a) Results of GeneScan analysis in patients with high-grade multicentric lymphoma (Cases 3, 6 and 7). The left and the right panels show the results of *immunoglobulin heavy-chain* (*IgH*) gene, and the middle panel shows the results of *T-cell receptor gamma-chain* (*TCRγ*) gene. (b) *TCRγ* in patients with gastrointestinal (GI) lymphoma (Cases 23, 26 and 29). (c) *TCRγ* in patients with cutaneous lymphoma (Cases 30 and 32). LN: lymph node, Duo: duodenum, bp: base pairs.

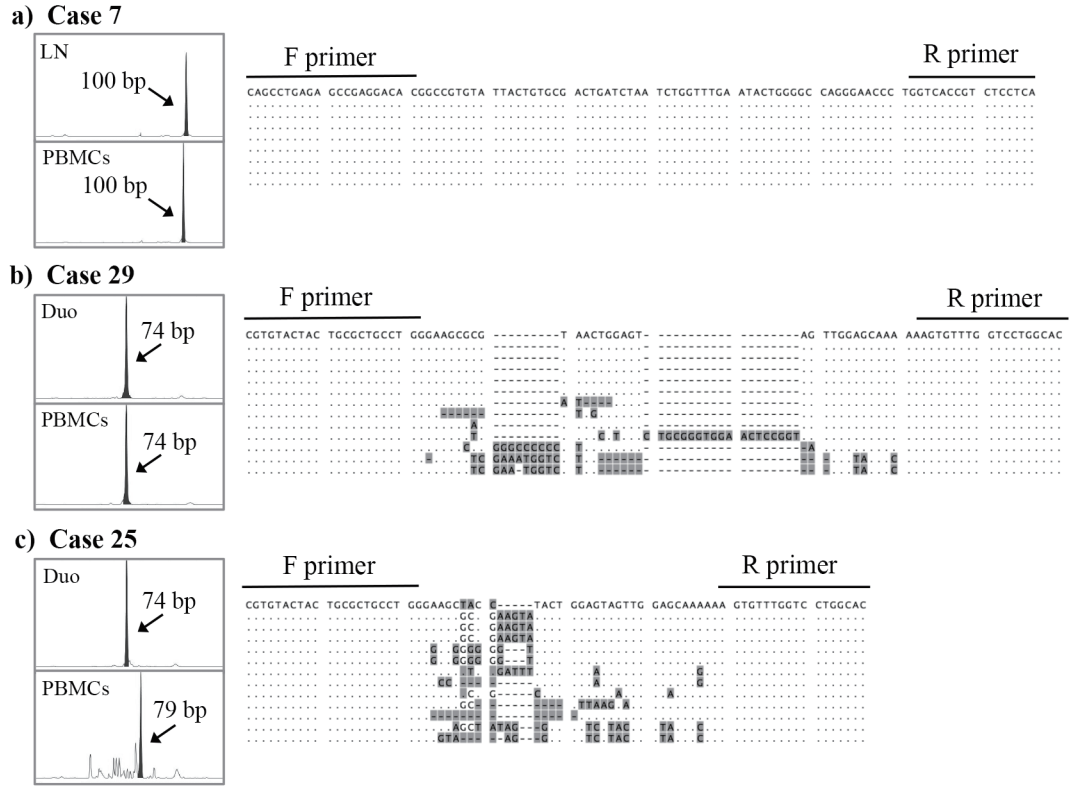

Fig. 2. Sequence comparison of the PCR products of primary lesions and peripheral blood mononuclear cells (PBMCs). The line at the top of each alignment shows the sequence from the primary lesions, and the alignment below represents the sequences analyzed from PBMCs. Nucleotide residues identical to the sequence of the lesional sample are depicted as dots in the PBMC sequence, and the background of minor nucleotide residues is shadowed. (a) Sequence of the *immunoglobulin heavy-chain* (*IgH*) gene in a patient with high-grade multicentric lymphoma in which clonal PCR products of identical size were detected between the samples (Case 7). The sequences were identical between lymph node (LN) and PBMCs, indicating the presence of CTCs. (b) Sequence of *T-cell receptor gamma-chain* (*TCRγ*) gene in a patient with gastrointestinal (GI) lymphoma in which clonal PCR products of identical size, between samples, were detected (Case 29). A sequence identical to the duodenal sample was found in the PBMC sample, indicating the presence of CTCs. (c) Sequence of *TCRγ* in a patient with GI lymphoma in which clonal PCR products of different sizes between samples were detected (Case 25). No sequence identical to lesional samples was detected in PBMCs. Duo: duodenum, bp: base pairs, F primer: forward primer, R primer: reverse primer.

PBMCs of five other dogs (42%) with GI lymphoma; however, the nucleotide lengths of the PCR products varied between primary lesions and PBMCs. Samples from a representative case were further subjected to CDR3 sequencing (Case 25). No sequence that was identical to duodenal samples was detected in 12 PBMC-derived clones analyzed (Fig. 2c).

Different clonal PCR products between primary lesions and PBMCs were specifically detected in dogs with GI lymphoma. In humans, some kinds of autoimmune diseases and food allergy result in clonal expansion of lymphocytes in peripheral blood [1]. Furthermore, clonal rearrangement of antigen receptor genes was demonstrated in a dog with ehrlichiosis [2]. Although dogs with inflammatory diseases, such as inflammatory bowel disease (IBD), were excluded from the present study, inflammatory changes of the intestine are often accompanied with GI lymphoma. Therefore, GI lymphoma cases concomitant with chronic enteritis were possibly included in this study and may be one possible reason for this phenomenon. This indicates that positive results in peripheral blood do not always indicate the presence of CTCs when using consensus primers in PARR. Since the GeneScan analysis can provide an accurate comparison of nucleotide size length, this technique is more advantageous for detecting the presence of CTCs compared to conventional PAGE in canine lymphoma patients.

Taking the advantage of GeneScan over the conventional PAGE, we compared the detection rate of CTCs among different anatomical subtypes of lymphomas. In the current study, the detection rate of CTCs in dogs with GI lymphoma was lower than in dogs with multicentric lymphoma. CTC levels in peripheral blood are reportedly observed in parallel with changes in total lymph node volumes, indicating that CTC volumes reflect tumor burden [24]. In the present study,

total tumor burden was smaller in GI lymphoma compared with multicentric lymphoma at the time of diagnosis. We speculated that patients were probably presented to the hospital in an early stage of tumor development, since even localized tumors in the GI tract are more likely to cause intensive and readily identifiable symptoms compared with peripheral lymph nodes.

On the other hand, for all of the multicentric lymphoma patients with positive results in peripheral blood, PCR product size was the same between peripheral blood and corresponding primary lesions. This might suggest that conventional PAGE would be sufficient for detecting CTCs at the time of diagnosis in dogs with multicentric lymphoma.

Two out of three dogs with cutaneous lymphoma were positive for CTCs in the current study. Although CTC detection in canine cutaneous lymphoma has not been previously investigated, there are several studies describing the significance of CTC detection in human cutaneous lymphoma [6, 20, 22]. One study detected peripheral blood T-cell clones by PARR using consensus primers as an independent prognostic marker in patients with mycosis fungoides [6]. Although only three dogs with cutaneous lymphoma were included in this study, further studies might reveal the clinical significance of CTC detection in dogs with cutaneous lymphoma.

This study has several limitations. First, lack of sequence confirmation in all cases. Second, there is possibility of PARR product of different size still representing neoplastic clone, as we conducted GeneScan analysis only in the single primary sample for each patient.

In conclusion, we show that 59.4% of dogs with lymphoma had CTCs as detected by GeneScan analysis, and the detection rate varied among the anatomical subtypes of lymphomas studied. The GeneScan analysis employed in this study could facilitate further studies on CTCs in dogs with lymphoma. However, determining the clinical significance of CTC monitoring on prognosis or relapse prediction using this novel technology requires further investigation.

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