doi: 10.17221/100/2016-VETMED

Pax5 as a potential candidate marker for canine B-cell lymphoma

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ABSTRACT: Immunophenotyping is a valuable method for prognosis in canine malignant lymphoma. The general B-cell marker is CD79a; however, Pax5 or B-cell specific activator protein, a transcription factor that controls B-cell identity and cell maturation, could also be used as a B-cell indicator in canine lymphomas. This study aimed to use Pax5, CD79a and CD3 expression in immunohistochemistry of spontaneous canine lymphomas, in order to carry out diagnosis and histopathological classification according to the World Health Organization guidelines. Forty-six retrospective cases including 33 multicentric, eight extranodal, and five alimentary lymphomas in dogs were immunostained by anti-Pax5 and anti-CD79a antibodies for B-cell identification, and anti-CD3 antibody for T-cell identification. T-cell lymphomas (CD3+/Pax5–/CD79a–) accounted for 30.43% of cases (14/46), and four of the lymphomas (28.57%) presented with CD3+/Pax5–/CD79a+. Conversely, B-cell lymphomas (CD3–/Pax5+/CD79a+) accounted for 69.57% of cases (32/46) and 12.5% of these (4/32) showed only Pax5-positive cells (CD3–/Pax5+/CD79a–). Therefore, in dogs, Pax5 appears to be a more useful marker for staining all B-cell subtypes compared to CD79a. Immunophenotyping with both Pax5 and CD3 are necessary for lymphoid lineage identification in canine lymphomas.

Keywords: B-cell marker; dog; immunohistochemistry; lymphoma

Malignant lymphoma is a common hematopoietic tumour in animals, especially in dogs. Canine lymphoma was found to represent approximately 1.94% of all canine tumours and 76.6% of all hematopoietic neoplasms in Bangkok, Thailand (Rungsipipat et al. 2012), similarly as in other countries (Weiss 2006; Regan et al. 2013). Canine malignant lymphomas are normally derived from a clonal expansion of neoplastic B or T lymphocytes. B-cell lymphomas have a greater incidence rate (60–80%) than T-cell lymphomas (10–38%). However, mixed B- and T-cell lymphomas and null-cell type lymphomas have also been reported (Wilkerson et al. 2005). Many studies have reported that T-cell lymphomas are characterised by shorter survival times and disease-free intervals than lymphomas of B-cell origin (Ponce et al. 2004; Valli et al. 2013). Because of this dissimilar prognosis, histopathological classification with immunophenotyping is important for informing chemotherapeutic treatment in canine lymphomas (Rebhun et al. 2011). Histopathological classification, for example, the updated Kiel (Fournel-Fleury et al. 1997; Fournel-Fleury et al. 2002) or World Health Organization classifications (Valli et al. 2011), uses criteria based on the immunophenotype, cell morphology, and tis-

Supported by the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University, Thailand (CU-57-001-HR) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), Thailand. SS was supported by the Overseas Presentations of Graduate Level Academic Thesis Scholarship and the Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of the birthday of his Majesty King Bhumibol Adulyadej.

doi: 10.17221/100/2016-VETMED

sue architecture. Immunophenotyping data was also shown to be related to survival (Valli et al. 2013).

Gold standard methods, such as immunohistochemistry (IHC) or immunocytochemistry, have been developed for immunophenotyping classification in humans, dogs and cats. The typical protein markers for identification of B and T lymphocytes are CD79a and CD3, respectively (Ferrer et al. 1993; Ponce et al. 2004; Fernandez et al. 2005; Vezzali et al. 2010; Valli et al. 2011). *Pax5* is a member of the paired-box domain family of transcription factors that encodes the B-cell-specific activator protein. Its important roles are to control B-cell identity, development and differentiation. Pax5 protein is expressed in normal and neoplastic cells from the pro-B to mature B-cell stages (Horcher et al. 2001). It serves as a pan pre B-cell marker and was shown to be more specific than CD79a (Willmann et al. 2009). In human studies, Pax5 expression was restricted to B-cell malignancies including those that lacked CD20 and CD79a expression (Jensen et al. 2007). To the best of our knowledge, only a few studies have investigated Pax5 expression in canine lymphoma using IHC. The aim of this study was to perform immunophenotyping on canine lymphoma cases using IHC techniques and staining for CD3, a T-cell marker and for the B-cell markers, CD79a and Pax5.

MATERIAL AND METHODS

Tissue samples. Thirty-four samples from biopsies and 12 samples from necropsies were submitted to the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University during the period 2008–2013. Patients presented with clinical signs of generalised lymphadenopathy, abdominal enlargement, or with chronic gastrointestinal signs. Thoracic radiography, abdominal radiography and abdominal ultrasonography were performed for anatomic classification. Thirty-three multicentric (at least clinical stage III), five alimentary, and eight extranodal (skin, liver, spleen, tongue, heart) lymphomas were included in this study. Tissue samples were fixed in 10% buffered formalin, routinely histologically processed, paraffin-embedded, stained with H&E and examined under a light microscope.

Histopathology. Histopathological changes of cell size and shape, nucleus size, mitotic index, number of nucleoli, chromatin density, cytoplasm characterisation of neoplastic lymphocytes and tissue architecture, were evaluated based on the World Health Organization classification by a veterinary pathologist (Vezzali et al. 2010; Valli et al. 2011). The number of mitoses per five high-power fields was noted as the mitotic index.

Immunohistochemistry. Four to six µm-thick sections were immunophenotypically classified using anti-CD3, anti-Pax5, and anti-CD79a, to identify T- and B-cell lineages, respectively. The immunohistochemical protocol was modified from Rungsipipat et al. (2012). Briefly, antigen retrieval for CD3 was achieved by heating slides with 10mM citrate buffer (pH 6.0) in a microwave oven. To block endogenous enzymes, the slides were incubated in 3% H_2O_2 for 10 min and 1% bovine serum albumin at 37 °C for 10 min, respectively. After washing the slides with 0.1M phosphate buffer solution (pH 7.4), they were incubated with ready-to-use monoclonal mouse anti-human CD3 antibody (LN 10, Leica, UK) at 4 °C for 12–14 h. After washing, the slides were incubated with modified streptavidin-biotin-peroxidase complex or Envision polymer (Dako, Denmark) at room temperature for 45 min. Finally, they were immersed in 3,3'-diaminobenzidine to develop the immunological reaction and counterstained with Mayer's haematoxylin before mounting.

Similarly, to determine B-cell lineages immunostaining with anti-Pax5 (clone 1EW, Leica, UK) was performed. Following deparaffinisation and dehydration with xylene and a graded series of alcohol, antigens were retrieved by heating in Tris/EDTA (pH 9.0) in an autoclave oven (121 °C, 5 min), followed by incubation with monoclonal mouse anti-human Pax5 antibody (dilution 1 : 50) at 4 °C overnight. Slides were then rinsed with phosphate buffer solution; endogenous peroxidase was blocked with 3% H_2O_2 at room temperature for 10 min and non-specific background was blocked using 1% bovine serum albumin at 37 °C for 10 min. The LSAB technique or Novolink detection system (Leica, UK) was used for conjugation to tissues at room temperature for 15 min. 3,3'-diaminobenzidine was used as a chromogen and Mayer's haematoxylin was used for counterstaining.

CD79a was used as further B-lymphocyte marker. Citrate buffer pH 6.0 was used to retrieve antigens in an autoclave at 121 °C for 5 min. After blocking steps (3% H_2O_2 at room temperature for 10 min and 1% bovine serum albumin at 37 °C for 10 min), monoclonal mouse anti-human CD79a (clone HM57,

doi: 10.17221/100/2016-VETMED

Dako, Denmark) diluted to 1 : 100 was incubated with sections at 4 °C for 12–14 h. Then, sections were incubated with Novolink polymer (Leica, UK) at room temperature for 15 min, lastly; colour was developed with 3,3'-diaminobenzidine, and sections were counterstained with Mayer's haematoxylin, and mounted with Permount (Fisher Scientific, USA).

A normal lymph node from a dog necropsy was used as an antibody control. A positive identification of a B-cell lymphoma was made when at least 60% of neoplastic cells were positively stained with CD79a in their cytoplasms and with Pax5 in their nuclei. Identification of T-cell lymphomas was made when at least 60% of neoplastic cells were primarily stained with CD3 on the membrane of the T-cells (Willmann et al. 2009).

RESULTS

In this study, out of a total of 46 dogs, 23 were males and 23 were females. Both pure (32/46) and mixed (14/46) breeds were included. The major purebreeds were Golden retriever (9/32), Poodle (7/32), and Shih Tzu (4/32). The median age was eight years old (range 3–15 years; Table 1). Based on anatomical classifications, multicentric lymphoma was diagnosed in 71.74% of cases (B-cell = 27/33 and T-cell = 6/33), intestinal lymphoma in 10.87% of cases (B-cell = 1/5 and T-cell = 4/5), and extranodal lymphoma in 17.39% of cases (B-cell = 4/8 and T-cell = 4/8). Additionally, 58.7% (27/46) of the canine lymphomas were categorised as high-grade. High-grade B-cell lymphomas represented 68.75% of cases (22/32; Table 2). The common histopathologies of high-grade B- and T-cell lymphomas in this study were diffuse large B-cell lymphomas (Figure 1), which consisted of centroblastic and immumoblastic cells, and peripheral T-cell lymphomas, which composed of pleomorphic small to large-sized cells, respectively. The less common low-grade lymphomas were follicular lymphomas (Figure 2), T-cell small lymphocytic lymphomas (Figure 3) and cutaneous T-cell lymphomas (Figure 4). In addition, MI was on average 8/HPF in high-grade and 4/HPF in low-grade lymphomas.

Immunophenotyping using IHC revealed positive Pax5 staining in the nuclei of all B lineages (Figures 1B and 2B) as well as CD3 staining in the cytoplasm of T-lymphocytes in all cases (Figures 3C and 4C). However, CD79a staining gave a negative result in four cases of B-cell lymphoma (Dog No. 1, 2, 20, 42) and a positive result in four cases of T-cell lymphoma (Dog No. 33, 35, 36, 38) as shown in Table 1. Two from three follicular lymphomas were

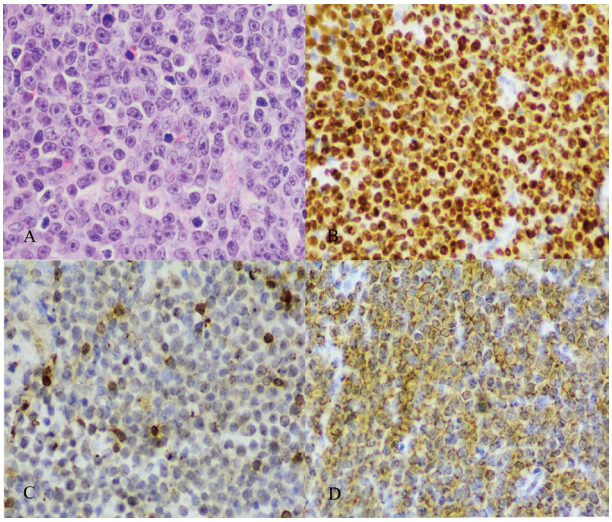

Figure 1. Diffuse large B-cell lymphoma. (**A**) Histopathology showed large neoplastic lymphocytes with round-to-cleaved nuclei, prominent nucleoli and abundant cytoplasm; H&E, × 400. (**B**) Positive Pax5 staining in the nuclei of B-cells; immunohistochemistry (IHC), × 400. (**C**) Neoplastic cells showed negative results in CD3 staining; IHC, × 400. (**D**) CD79a antibodies revealed positive immunolabelling on the cytoplasmic border of B cells; IHC, × 400

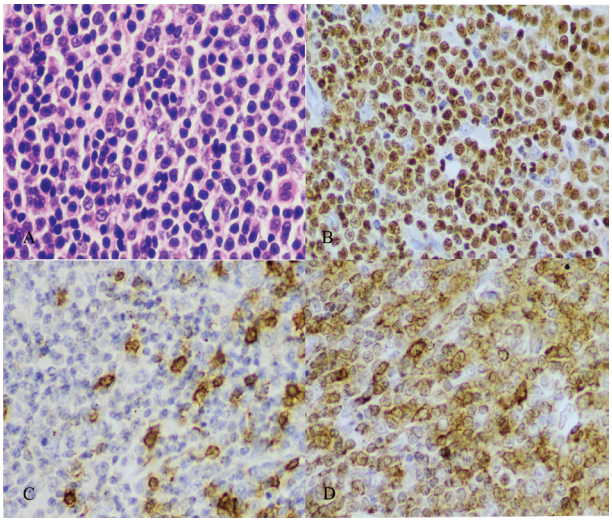

Figure 2. Follicular lymphoma. (**A**) Histopathology showed medium-sized and large-sized neoplastic lymphocytes; H&E, × 400. (**B**) Positive Pax5 staining in the nuclei of B-cells; immunohistochemistry (IHC), × 400. (**C**) Neoplastic cells showed some positive CD3 staining; IHC, × 400. (**D**) CD79a staining revealed positivity cell in the cytoplasm of B-cells; IHC, × 400

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Table 1. Signalment, anatomical location (AL), histological classification (HC), and immunohistochemistry detection in 46 canine lymphomas between 2008 and 2013

No.	Breed[a]	Age (years)	Sex[b]	AL	HC	Immunophenotype			No.	Breed[a]	Age (years)	Sex[b]	AL	HC	Immunophenotype		
						Pax5	CD79a	CD3							Pax5	CD79a	CD3
1	mixed	na	F	MC	B-SLL	+	–	–	24	GR	9	M	MC	DLBCL	+	+	–
2	CP	8	F	MC	B-SLL	+	–	–	25	MP	10	F	MC	DLBCL	+	+	–
3	mixed	11	M	H	LPL	+	+	–	26	Poodle	12	F	MC	DLBCL	+	+	–
4	LR	7	M	MC	B-SLL	+	+	–	27	Shih Tzu	na	M	MC	DLBCL	+	+	–
5	Shar Pei	5	F	H	HSTL	–	–	+	28	Shih Tzu	12	F	MC	DLBCL	+	+	–
6	mixed	9	F	A	ITCL	–	–	+	29	Shih Tzu	7	M	MC	DLBCL	+	+	–
7	GR	8	M	MC	T-SLL	–	–	+	30	mixed	7	M	MC	DLBCL	+	+	–
8	Poodle	na	F	MC	B-SLL	+	+	–	31	CP	14	F	MC	DLBCL	+	+	–
9	mixed	8	F	SP	FL	+	+	+[c]	32	mixed	10	M	MC	DLBCL	+	+	–
10	Poodle	na	M	MC	FL	+	+	+[c]	33	mixed	7	F	SP	HSTL	–	+	+
11	mixed	13	F	MC	FL	+	+	–	34	Boxer	3	M	A	ITCL	–	–	+
12	Schnauzer	7	M	MC	NMZ	+	+	–	35	BT	3	M	MC	PTCL	–	+	+
13	LR	10	F	MC	NMZ	+	+	–	36	GR	8	M	MC	PTCL	–	+	+
14	Poodle	9	M	A	ITCL	–	–	+	37	mixed	6	M	MC	PTCL	–	–	+
15	BT	10	M	MC	PTCL	–	–	+	38	GR	na	F	T	CTCL	–	+	+
16	Shih Tzu	12	F	A	DLBCL	+	+	–	39	mixed	4	M	C	CTCL	–	–	+
17	Dachshund	na	M	SP	DLBCL	+	+	–	40	GR	15	F	MC	PTCL	–	–	+
18	GR	na	F	MC	DLBCL	+	+	–	41	Poodle	11	M	MC	B-LBL	+	+	–
19	mixed	8	M	MC	DLBCL	+	+	–	42	mixed	8	M	MC	B-LBL	+	–	–
20	GR	4	F	MC	DLBCL	+	–	–	43	GR	na	F	A	ITCL	–	–	+
21	mixed	12	M	MC	DLBCL	+	+	–	44	Poodle	7	F	MC	B-LBL	+	+	–
22	Poodle	9	M	MC	DLBCL	+	+	–	45	GR	12	F	MC	B-LBL	+	+	–
23	BT	7	F	MC	DLBCL	+	+	–	46	mixed	6	F	HB	ALBL	+	+	–

A = alimentary, ALBL = anaplastic large B-cell lymphoma, B- or T-SLL = B-cell or T-cell small lymphocytic lymphoma, B-LBL = B-lymphoblastic lymphoma, BT = Bull terrier, C = cutaneous, CP = Cocker spaniel, DLBCL = diffuse large B-cell lymphoma, F = female, FL = follicular lymphoma, GR = Golden retriever, H = hepatic, HB = heart base, HSTL = hepatosplenic T-cell lymphoma, ITCL = intestinal T-cell lymphoma, LPL = lymphoplasmacytic lymphoma, LR = Labrador retriever, M = male, MC = multicentric, MP = Miniature pinscher, na = data not available, NMZ = nodal marginal zone lymphoma, PTCL = peripheral T-cell lymphoma, SP = splenic, T = tongue

[a]32 purebreeds, 14 mixed breeds

[b]23 males, 23 females

[c]Normal T lymphocytes showed positive staining in the pericortical area

mainly reactive with Pax5 and CD79a in the follicle area, and were stained moderately with CD3 in the paracortical area. Pearson's chi-squared test also revealed an association between immunophenotyping and histological grade ($P = 0.036$).

DISCUSSION

Canine lymphoma in this study was classified into three anatomical locations: multicentric, alimentary, and extranodal, multicentric lymphomas have frequently been observed in dogs (Dobson et al. 2002; Rungsipipat et al. 2012). In this study, B-cell lymphoma was found in 69.57% of cases, whereas T-cell lymphoma was detected in the remaining 30.43%. This higher incidence rate of B-cell lymphoma is similar to that reported previously (Fournel-Fleury et al. 2002; de Arespacochaga et al. 2007). Pax5 protein expression was observed in all B-derived lymphoma samples, while CD79a reactivity was not observed in four cases of B-cell lymphomas. Willmann et al. (2009) also described this problem. Moreover, four T-cell lymphomas

doi: 10.17221/100/2016-VETMED

Table 2. Histopathological classification and immunophenotyping based on the World Health Organization classification

B-cell	No. of cases	T-cell	No. of cases
Low grade: 31.25% (10/32)		**low grade**: 64.29% (9/14)	
B-cell small lymphocytic lymphoma	4	T-cell small lymphocytic lymphoma	1
Llymphoplasmacytic lymphoma	1	hepatosplenic T-cell lymphoma	2
Follicular lymphoma	3	intestinal T-cell lymphoma	4
Nodal marginal zone lymphoma	2	cutaneous T-cell lymphoma	2
High grade: 68.75% (22/32)		**high grade**: 35.71% (5/14)	
Diffuse large B-cell lymphoma	17	peripheral T-cell lymphoma	5
B-cell lymphoblastic lymphoma	4	total	14
Anaplastic large B-cell lymphoma	1		
Total	32		

expressed both CD3 and CD79a, which has been reported previously in both humans and dogs (Wilkerson et al. 2005; de Arespacochaga et al. 2007; Willmann et al. 2009). Additionally, follicular B-cell lymphomas showed positive staining with CD3, which might be a result of reactive T-cell population involvement. Although the Pax5 marker is superior to CD79a in B-cell identification, CD79a is still frequently selected for immunophenotyping in dogs with lymphoma (Vezzali et al. 2010; Valli et al. 2013). Both markers have high correlations through various B lymphocyte stages; however, Pax5 is expressed only during B-cell development and differentiation, and not in plasma cells (Horcher et al. 2001). Agostinelli et al. (2010) reported that DAK-Pax5 (clone 24) staining resulted in more intensely positive cells in most canine B-non-Hodgkin lymphomas apart from plasmacytomas. Nevertheless, the limitation of this antibody and IHC protocol might be a problem of its extensive application in animals.

In humans, Pax5 expression has been observed in B-lymphoblastic leukaemia/lymphoma, small lymphocytic lymphoma, diffuse large B-cell lym-

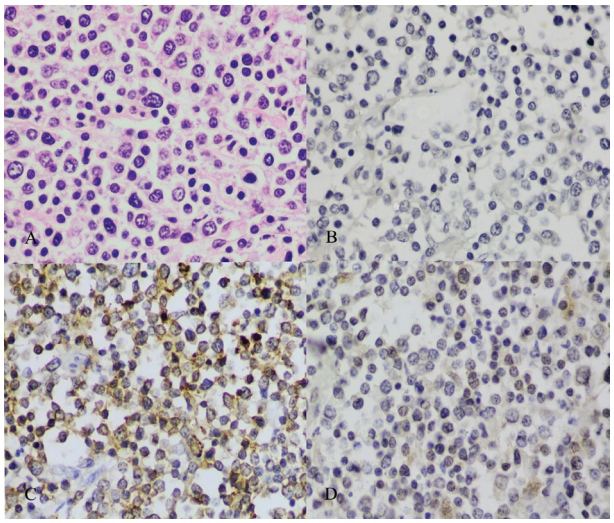

Figure 3. T-cell small lymphocytic lymphoma. (**A**) Histopathology showed small lymphoblasts with pleomorphic nuclear size and scant basophilic cytoplasm; H&E, × 400. (**B**) Negative immunolabelling with Pax5 antibody; immunohistochemistry (IHC), × 400. (**C**) Neoplastic cells showed intense positive immunostaining with CD3 antibody; IHC, × 400. (**D**) CD79a antibodies gave negative results; IHC, × 400

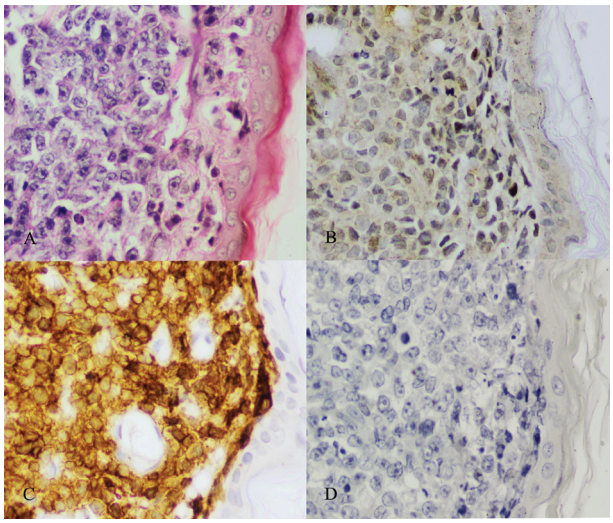

Figure 4. Cutaneous T-cell lymphoma. (**A**) Histopathology showed large lymphoblasts with pleomorphic nuclear size and moderate amounts of basophilic cytoplasm; H&E, × 400. (**B**) Negative results with Pax5 antibody; immunohistochemistry (IHC), × 400. (**C**) Neoplastic cells showed positive staining with CD3 antibody; IHC, × 400. (**D**) CD79a antibodies gave negative results; IHC, × 400

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phoma, Burkitt lymphoma, Mantle cell lymphoma, Hodgkin lymphoma and non-Hodgkin lymphoma (Browne et al. 2003; Desouki et al. 2010; Nasr et al. 2010). Furthermore, Pax5 expression was detected in B-cell lymphoma which occasionally lacks CD20 and CD79a expression (Jensen et al. 2007). Thus, Pax5 is accepted as a B-cell marker in human medicine. In the veterinary field, Pax5 was chosen as a B-cell indicator in canine malignant lymphoma because of its specificity and sensitivity; CD79a-positive B-cells were reported to be expressed in 10–40% of canine and human T-cell lymphomas (Willmann et al. 2009). Pax5 staining was used for B-cell identification in a complicated case report on multicentric B-cell lymphoma with neurolymphocytosis (Schaffer et al. 2012). Pax5 was also employed in a study of inflammatory processes: spirocercosis-induced nodular formation in dogs (Dvir et al. 2011), and was also used in an immunocytochemistry by liquid-based cytology and tissue transfer technique for immunophenotyping in canine non-Hodgkin lymphoma (Stone and Gan 2014; Fernandes et al. 2015). The sensitivity and specificity of Pax5 immunohistochemistry for B-cell lymphomas when compared to heteroduplex polymerase chain reaction for antigen receptor rearrangements of IgH primer sets for B-cells, were 48% and 100%, respectively (Sirivisoot et al. 2016). In our study, Cases No. 1, 2, 20 and 42 were diagnosed as B-cell lymphomas because significant numbers of Pax5-positive B-cells were present.

One complication to staining for Pax5 is the difficulty of choosing a suitable clone of Pax5 antibody for canine tissues. At first, we used DAK-Pax5 monoclonal anti-human antibody, but it failed to stain canine B lymphocytes. A previous study reported that a few cases of B-non-Hodgkin lymphomas and classical Hodgkin lymphomas from human and animal tissues showed negativity with this DAK-Pax5 clone (Agostinelli et al. 2010). However, when we used the anti-human Pax5 monoclonal antibody clone 1EW, this stained both normal and neoplastic B-cells. This discrepancy might occur due to species-specific differences, different epitopes, and the differing cross-reactivity of each antibody. The 1EW clone had 96–98% homologous identity with the canine epitope, similar to CD3 clone LN10 and CD79a clone HM57, which showed cross reactivity to canine antigens.

The immunophenotyping technique provides reliable information on the clonal origin of canine malignant lymphomas. Because of the pronounced dissimilarities in prognosis and treatment of B- and T-cell lymphomas, IHC is necessary for lineage determination. Besides its usefulness in precisely marking lymphomas of B-cell origin, Pax5 staining might serve as a suitable diagnostic tool in unclassified lymphomas in dogs and also in other species.

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Received: June 14, 2016
Accepted after corrections: December 14, 2016