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Original article

# Immunohistochemical study of expression of immunoglobulins in canine B-cell lymphomas

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## Abstract

Nineteen canine lymphomas were included in this study. Tumors were classified according to the updated Kiel classification adapted for canine lymphomas by Fournel-Fleury et al. Immunoglobulin light chains ( $\kappa$  and  $\lambda$ ) and IgM and IgG expression were determined by immunohistochemical method. In all examined cases neoplastic cells were positive for one of the immunoglobulin light chains. Expression of  $\lambda$  light chains and  $\kappa$  light chains was observed in 18/19 and 1/19 tumors, respectively. In the majority of neoplastic cells in each examined specimen this reaction had a membranous pattern ( $s\kappa/s\lambda$ ). In all examined cases the presence of immunoglobulin light chains was also observed in the cytoplasm of some neoplastic cells ( $c\kappa/c\lambda$ ). These cells were usually rare and never constituted a dominant population. The expression of immunoglobulin was found in 13/19 cases. Most lymphomas were sIgM positive (11/13 cases). In one case expression of IgG was found, and in another lymphoma two populations of neoplastic cells with different expression of examined immunoglobulins (cells with IgM<sup>+</sup> and IgG<sup>+</sup> phenotypes) were observed. The reaction also had a membranous pattern. The cells containing cytoplasmic immunoglobulins were rare, and in most cases were of the same type as the surface immunoglobulins. Our study has confirmed that canine lymphomas are a monoclonal proliferation of B-cells usually expressing immunoglobulin  $\lambda$  light chains and that the vast majority of tumors deriving from B-cells express IgM. Our study also indicates a possibility of occurrence of biclonal lymphomas in canine species.

**Key words:** dog, lymphoma, immunoglobulin light chains, immunoglobulin

## Introduction

Tumors arising from hematopoietic tissue are one of the most common canine tumors. Lymphomas are the most frequently diagnosed form of hematopoietic neoplasms. The term malignant lymphoma (*lymphoma malignum*) denotes neoplastic proliferation

of lymphocytes arising from any stage of their maturation and differentiation, from precursors to effector cells.

In human medicine, diagnosis and classification of lymphomas are made taking into account inclusively morphology and phenotype of neoplastic cells, their genetic features and clinical data (Harris et al. 2001).

One of the most important steps in the histopathological diagnosis of lymphomas is phenotype determination on the basis of the panel of specific antibodies. Unfortunately, estimation of the phenotype of canine lymphomas is still limited by the paucity of specific antibodies recognizing canine lymphoid antigens.

Immunoglobulins and immunoglobulin light chains are the markers of B lymphocytes, used in lymphoma diagnostics.

Demonstration of light chain expression on lymphocytes is not only a way of identifying B cells, but also, and perhaps above all, it is the most important marker used to distinguish neoplastic monoclonal proliferation from reactive hyperplasia of B cells. It is well known that each B lymphocyte produces one kind of immunoglobulin which, depending on its class, consists of one heavy chain ( $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\mu$ ) and one out of two light chains, either  $\lambda$  or  $\kappa$ . In the case of reactive hyperplasia, there is a polyclonal population of B cells restricted to lambda or kappa light chain production. Demonstration of a monoclonal population of lymphocytes producing only one type of light chain almost always indicates a neoplastic process (Levy et al. 1977, Mioduszezewska 1998). In humans, the ratio of kappa to lambda bearing B cells in lymphoid tissue is 2:1. Any deviation from this physiological balance indicates a clonal proliferation of lymphocytes (Levy et al. 1977). Compared with humans, in animals, including dogs, the physiological light chain ratio is largely dominated by the lambda chains (Arun et al. 1996).

Other B cell lineage markers are immunoglobulin molecules. They are located on the surface or in the cytoplasm of lymphocytes. Their localisation, class and intensity of expression depend on the stage of maturation and differentiation process of a given B cell. IgM, immunoglobulin with a  $\mu$  heavy chain is present on immature B cells, naive lymphocytes including B cells in the primary follicles and in mantle of the secondary follicles (surface immunoglobulins), and in plasma cells taking part mainly in primary immune response (cytoplasmic immunoglobulins). IgG, immunoglobulin with a  $\gamma$  heavy chain is present on B cells involved in the secondary immune response, including germinal center cells of the secondary follicles (surface immunoglobulins) and plasmocytes (cytoplasmic immunoglobulins) (Ferry and Harris 1997). Demonstration of a given immunoglobulin expression in lymphoma cells, in particular determination of its localisation (surface/cytoplasmic) in conjunction with other data on their phenotype and morphology, is used in a differentiate diagnosis of certain types of lymphomas with similar morphology.

There are just a few studies on the expression of immunoglobulin light chains and immunoglobulin molecules on canine lymphoma cells. Thus, the aim of this study was evaluation of the expression of these markers in canine lymphomas.

## Materials and Methods

Nineteen popliteal lymph nodes with lymphoma of B-cell origin were included in this study. The material was collected by surgical biopsy from dogs with suspected multicentric lymphoma.

All specimens were fixed in 10% neutral buffered formalin and processed by common paraffin technique. Tumors were classified according to the updated Kiel classification adapted to canine species by Fournel-Fleury et al. (1997).

Immunoglobulin light chains and IgM and IgG expression were determined immunohistochemically using the following polyclonal antibodies: anti-kappa light chains, anti-lambda light chains, anti-IgM and anti-IgG (Dako, Carpinteria, CA, USA).

All immunohistochemical procedures were performed according to the manufacturer's protocols. Antigens were unmasked either by twice microwaving in citrate buffer (immunoglobulin light chains) or by incubation with proteinase K (IgM, IgG). Sections were then incubated with primary antibody (diluted 1:1000, 1:150, 1:250 for immunoglobulin light chains, IgM and IgG respectively) for 1 hour at room temperature. The En Vision+™ Peroxidase® (Dako) visualization system was used for antigen detection.

Reactive canine lymph nodes were used as a positive control and substitution of primary antibody by Tris Buffered Saline was employed for negative controls.

For each pair of antibodies (kappa and lambda light chains as well as IgM and IgG) the dominant cell population was determined. The number of cells reacting with the latter antibody was estimated semiquantitatively using a scale from + to ++++. The cellular staining pattern, membranous or cytoplasmic (surface/cytoplasmic immunoglobulins, s/c), was defined and the dominant expression pattern was determined. Additionally, the number of cells showing the opposite staining pattern were semiquantitatively scored using a scale from + to ++++.

## Results

Clinical and pathological characteristics of 19 cases of B-cell lymphomas are presented in Table 1.

The B-cell lymphomas examined represented the following subtypes: immunoblastic – 1 case, centroblastic-centrocytic – 5 cases, centroblastic – 7 cases and Burkitt-like – 6 cases.

In each of the examined cases, almost all neoplastic cells were positive for one of the immunoglobulin light chains. This reaction has a membranous pattern ( $\kappa/\lambda$ ). All cases expressed  $\lambda$  light chain, except one tumor in which  $\kappa$  light chain expression was found (Table 2).

Table 1. Clinical and pathological characteristics of examined cases of B-cell lymphomas.

Case no.	Breed	Age (years)	Sex	Type of lymphoma	Clinical stage at time of biopsy
1	Mongrel	9	Female	Centroblastic-centrocytic	IIIa
2	Miniature schnauzer	7	Female	Centroblastic-centrocytic	IVb
3	Great Dane	7	Male	Centroblastic-centrocytic	IVb
4	Irish setter	8	Female	Centroblastic-centrocytic	IIIb
5	Mongrel	13	Female	Centroblastic-centrocytic	IIIa
6	Mongrel	12	Male	Immunoblastic	IVb
7	German shepherd	3	Female	Centroblastic	IIIa
8	Mongrel	13	Male	Centroblastic	IVa
9	Rhodesian ridgeback	4	Male	Centroblastic	IIIb
10	Bull terrier	7	Female	Centroblastic	IVb
11	Briard	4	Female	Centroblastic	IVb
12	Stafford	6	Male	Centroblastic	Vb
13	Mongrel	9	Female	Centroblastic	IIIa
14	Rottweiler	4	Male	Burkitt-like	IIIa
15	Rottweiler	6	Female	Burkitt-like	IIIb
16	Beagle	7	Male	Burkitt-like	IIIa
17	Mastiff	5	Female	Burkitt-like	IVb
18	Bullmastiff	9	Male	Burkitt-like	IVb
19	Doberman	12	Female	Burkitt-like	Vb

Table 2. Expression of surface immunoglobulins in examined morphological subtypes of B-cell lymphomas.

Morphological subtype	Number of cases	Light chain expression		Immunoglobulin expression		
		s $\lambda$	s $\kappa$	sIgM	sIgG	sIgMsIgG
Centroblastic-centrocytic	5	5/5	0/5	3/5	0/5	0/5
Immunoblastic	1	1/1	0/1	1/1	0/1	0/1
Centroblastic	7	7/7	0/7	4/7	0/7	1/7
Burkitt-like	6	5/6	1/6	4/6	1/6	0/6
<b>Total</b>	<b>19</b>	<b>18/19</b>	<b>1/19</b>	<b>11/19</b>	<b>1/19</b>	<b>1/19</b>

In all examined cases the presence of immunoglobulin light chains was also observed in the cytoplasm of neoplastic cells ( $\kappa/\lambda$ ). In one case, cells containing cytoplasmic light chains ( $c\lambda^+$ ) were quite abundant, but they were not a dominant population. In all other cases the positive cytoplasmic reaction with light chains was observed only in single cells.

In the majority of examined lymphomas in which the presence of cells containing cytoplasmic light chains was observed (18/19 cases), these cells expressed the same type of light chains as the main cell population with a membranous pattern of reaction. In 6 of 19 cases (including 5  $s\lambda^+$  lymphomas and

1  $s\kappa^+$  lymphoma), there were also a few cells showing the expression of the latter light chain in the cytoplasm.

In 7 of 18 cases of  $s\lambda^+$  lymphomas the number of cells containing cytoplasmic  $\lambda^+$  light chains was estimated as +. Six tumors contained a greater number of cells showing cytoplasmic pattern of  $\lambda$  light chain expression, estimated as ++. In the remaining 5 lymphomas, the presence of these cells was assessed as +++. Moreover, in 5 of 18 cases the presence of cells with  $s\kappa^+$  phenotype was observed. The number of these cells in 3 of 5 cases was estimated as +, and in the remaining 2 cases as ++ and +++, respectively.

Table 3. Expression of cytoplasmic immunoglobulin in examined morphological subtypes of B-cell lymphomas.

Morphological subtype	Number of cases	cλ			κκ			cIgM			cIgG		
		+	++	+++	+	++	+++	+	++	+++	+	++	+++
Centroblastic-centrocytic	5	2/5	1/5	2/5	0/5	1/5	0/5	1/5	1/5	1/5	0/5	0/5	1/5
Immunoblastic	1	1/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1
Centroblastic	7	3/7	3/7	1/7	2/7	0/7	1/7	4/7	0/7	0/7	1/7	0/7	1/7
Burkitt-like	6	2/6	2/6	2/6	1/6	1/6	0/6	2/6	3/6	0/6	0/6	0/6	1/6
<b>Total</b>	<b>19</b>	<b>8/19</b>	<b>6/19</b>	<b>5/19</b>	<b>3/19</b>	<b>2/19</b>	<b>1/19</b>	<b>8/19</b>	<b>4/19</b>	<b>1/19</b>	<b>1/19</b>	<b>0/19</b>	<b>3/19</b>

In the case of  $\kappa\kappa^+$  lymphoma, the number of  $\kappa\kappa^+$  cells was assessed as  $++$ . The presence of single  $c\lambda^+$  cells was also noted, estimated as  $+$ . The data are presented in Table 3.

The expression of IgM and/or IgG was found in 13 out of 19 examined cases (Table 2). As in the case of light chain expression, in all examined specimens, the vast majority of neoplastic cells showed a membranous pattern of reaction (sIgM/sIgG). The majority of cases of lymphomas were sIgM positive (11/13 cases). In one case expression of IgG was found, and in another two populations of neoplastic cells of different phenotype were observed: sIgM<sup>+</sup> B-cells constituted a larger part of tumor parenchyma and sIgG<sup>+</sup> B-cells formed irregular foci scattered through neoplastic tissue. Infiltrates of sIgG<sup>+</sup> B-cells were localized around lymph node trabecules and vessels. Both sIgG<sup>+</sup> and sIgM<sup>+</sup>sIgG<sup>+</sup> lymphomas were of  $s\lambda^+$  phenotype.

In the examined cases the presence of immunoglobulins in the cytoplasm of single cells was also observed (Table 3). In sIgM<sup>+</sup> lymphomas the cIgM<sup>+</sup> cells were found in all analyzed cases, their amount was estimated as  $+$ ,  $++$  and  $+++$  in 6, 4 and 1 cases, respectively. Moreover, in one sIgM<sup>+</sup> tumor, the presence of cIgG<sup>+</sup> was found, and their number was assessed as  $+++$ . This was a case of lymphoma with the largest number of B-cells containing cytoplasmic light chains. The lymphoma of sIgG<sup>+</sup> phenotype contained quite a large population of cIgG<sup>+</sup> cells (estimated as  $+++$ ), no cIgM<sup>+</sup> cells were observed in this case. In the tumor of sIgM<sup>+</sup>sIgG<sup>+</sup> phenotype, both examined immunoglobulins were observed in the cytoplasm of single cells, estimated as  $+$ .

In 4 of 6 cases of sIgM<sup>+</sup>sIgG<sup>+</sup> lymphomas no cells showing cytoplasmic expression of the examined immunoglobulins were observed. In one case (centroblastic lymphoma) cIgG<sup>+</sup> cells were quite numerous, estimated as  $+++$ , and in other one (Burkitt-like lymphoma) only single cells expressing cIgM were found, assessed as  $+$ .

## Discussion

Our results confirmed the monoclonal character of neoplastic proliferation of B-cells in all examined cases. They show that the vast majority of canine lymphomas express  $\lambda$  light chains, which accounted for 94.7% of all examined cases, whereas the monoclonal proliferation of  $\kappa^+$  neoplastic cells was found only in one case (5.3%). Similar results have been obtained in those few studies, which also examined light chain expression in canine B-cell lymphomas. Fournel-Fleury et al. (1997) have shown expression of  $\lambda$  light chain in 85.2% of 22 examined cases; however, the frequency of  $\kappa^+$  lymphomas was higher (14.8%) than in our study. In a study of cutaneous lymphoid neoplasms, expression of  $\lambda$  light chain was observed in about 60% of examined tumors (Sandusky et al. 1987). Similarly, most canine plasmocytomas express  $\lambda$  light chain, and  $\kappa^+$  neoplasms are much rarer. In one of the studies (Brunnert and Altman 1991) they constituted 24% of cases, whereas in another study (Cangul et al. 2002) all 63 examined cases of plasmocytoma were positive for  $\lambda$  light chain, whereas  $\kappa^+$  plasma cells were seen only in the cellular infiltration around tumors.

All examined cases of canine lymphomas showed a membranous pattern of light chains expression. The presence of cytoplasmic light chains is a characteristic feature of lymphoplasmocytic lymphoma and plasma cell neoplasms. Moreover, the variable number of tumor cells expressing cytoplasmic light chains is observed in some other morphological subtypes of lymphomas, including diffuse large B-cell lymphoma which is a large and heterogenous group of lymphoma (Mioduszevska 1998). This subtype of lymphoma, marked out in the latest lymphoma classifications (REAL, WHO) includes several different morphological variants, including centroblastic lymphoma, immunoblastic lymphoma and Burkitt-like lymphoma, i.e. the morphological subtypes identified in our study (Mioduszevska 1998, Gatter and Warnke 2001). The presence of single cells with cytoplasmic

light chain expression was observed in 8 of 19 examined cases. Surprisingly, in the research of Fournel-Fleury et al. (1997), among 5 lymphomas expressing cytoplasmic light chains, three were immunoblastic lymphomas (two others were classified as lymphoblastic lymphomas), whereas the only case of immunoblastic lymphoma identified in our study showed the presence of cytoplasmic  $\lambda$  light chain only in single cells.

More data is available on the expression of different immunoglobulin classes in canine lymphoma cells. In our study tumors with IgM expression were predominant (57.9%). In 2 cases (10.5%) neoplastic cells produced IgG. In the remaining 5 cases no expression of the examined immunoglobulins was found, which may indicate that these lymphomas produce one of the remaining immunoglobulin classes or only immunoglobulin light chains. Similarly, the results of other studies (Teske et al. 1994, Fournel-Fleury et al. 1997, Wilkerson et al. 2005) indicate that tumors expressing IgM are predominant among canine B-cell lymphomas, representing from 40% to about 70% of cases. Tumors expressing other immunoglobulin classes are much rarer, they constitute 10-20% of examined cases (Teske et al. 1994, Fournel-Fleury et al. 1997, Wilkerson et al. 2005). In the research of Wilkerson et al. (2005) all IgM<sup>+</sup> lymphomas expressed IgG. In a study conducted by Fournel-Fleury et al. (1997) expression of IgG was observed in 7 out of 10 IgM<sup>+</sup> tumors (2 of 3 remaining cases produced IgA; expression of IgD was not examined in this study). In lymphomas analysed by Teske et al. (1994), the percentages of tumors expressing IgG, IgA and IgD were similar (about 12% of examined cases).

Wilkerson et al. (2005) did not classify the analysed tumours morphologically, while all cases of canine B-cell lymphomas showing expression of immunoglobulin other than IgM, included in the papers quoted above (Teske et al. 1994, Fournel-Fleury et al. 1997) were classified as immunoblastic or centroblastic lymphomas. In our study IgG<sup>+</sup> tumors were classified as centroblastic and Burkitt-like subtypes.

In one of two lymphomas positive for IgG examined in our study IgG expression coexisted with the presence of IgM. The phenomenon of two different immunoglobulin classes being produced by lymphoma cells was also observed in individual cases by other authors (Teske et al. 1994, Fournel-Fleury et al. 1997) and not only in the case of IgG and IgM but also for other immunoglobulin classes (IgM and IgD, IgG and IgD). Among 5 cases of lymphoma showing coexistence of two classes of immunoglobulins observed by Teske et al. (1994), four were classified as centroblastic subtype. Similarly, the case of IgM and IgG positive tumor from our study was also a centroblastic lymphoma. However, in a quoted paper (Teske et al. 1994) the coexpression of two different immunog-

lobulin classes was observed, while in our study the presence of two separate populations of neoplastic cells producing different immunoglobulin classes was observed as IgM<sup>+</sup>IgG<sup>+</sup> lymphoma. This may indicate either the biclonal nature of this tumor or the occurrence of new lymphoma cell clones originating from the same neoplastic stem cell during tumor progression. Both these situations are observed in human lymphomas. Biclonal lymphomas represent a small percentage of these neoplasms (Sanchez et al. 2003). In such tumors the coexistence of two or more B-cell clones producing different immunoglobulin light and/or heavy chains are observed (Sanchez et al. 2006). The case of biclonal follicular lymphoma presenting two distinct neoplastic cell clones, one infiltrated lymph node, and the latter bone marrow, has been also described (Nakamura et al. 2009). The phenomenon of intraclonal evolution can also explain the presence of two separate lymphoma cell populations expressing different immunoglobulin classes (Ottensmeier and Stevenson 2000, Sanchez et al. 2006). The results of other studies suggest that isotype switch events can occur in lymphomas arising from germinal centers (Aarts et al. 2000, Ottensmeier and Stevenson 2000). As a result, new subsets of neoplastic B-cells producing different immunoglobulin classes within the original neoplastic clone may appear. This phenomenon has been observed in follicular lymphomas and diffuse large B-cell lymphomas (Aarts et al. 2000, Ottensmeier and Stevenson 2000). It may be that an isotype switch event occurred in the examined case, which was one of the centroblastic lymphomas and thus originated from germinal center cells. However, to confirm this molecular analysis of microdissected tissue with comparison of rearranged immunoglobulin sequences, as shown in man by Fend et al. (1999), would be necessary.

The results of our studies have confirmed that canine lymphomas are in most cases monoclonal populations of tumor cells expressing  $\lambda$  light chains, and that the vast majority of tumors deriving from B-cells express IgM. Our study also indicates the possibility of occurrence of biclonal lymphomas in canine species.

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