Immunohistochemical Identification of B and T Lymphocytes in Formalin-Fixed, Paraffin-Embedded Feline Lymphosarcomas: Relation to Feline Leukemia Virus Status, Tumor Site, and Patient Age

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ABSTRACT

The lymphocyte phenotype of 70 formalin-fixed, paraffin-embedded feline lymphosarcomas (LSAs) was determined immunohistochemically using a T cell polyclonal antibody, and a B cell monoclonal antibody. Forty-seven of 70 (67%) tumors were T cell, 19/70 (27%) were B cell, and 4/70 (6%) did not stain with either marker. Thirty-eight of 70 (54%) tumors were positive for feline leukemia virus (FeLV) antigen by immunohistochemistry (IHC), and 52/70 (74%) tumors were positive for FeLV DNA using the polymerase chain reaction (PCR). B cell tumors were as frequently FeLV-positive as T cell tumors using either IHC or PCR. Intestinal tumors were more likely to be B cell than T. The incidence of B and T cell tumors was not different among young (≤ 3 y), middle-aged (> 3 y to ≤ 8 y), and old (> 8 y) cats. Both B and T cell tumors from old cats were FeLV-positive more often by PCR than by IHC. Feline leukemia virus DNA but not antigen, was detected in B cell tumors and intestinal tumors from cats > 8 y as often as it was detected in B cell tumors and intestinal tumors from cats ≤ 8 y. Previously, most B cell and intestinal tumors from old cats were considered to be negative for FeLV. Here, the results suggest involvement of latent or replication-defective forms of the virus in such tumors from old cats. This study supports a role for FeLV in feline B cell as well as T cell tumorigenesis.

RÉSUMÉ

À l'aide d'un anticorps polyclonal anti-lymphocyte T et d'un anticorps monoclonal anti-lymphocyte B, le phénotype des lymphocytes retrouvés dans 70 lymphosarcomes félins a été déterminé par immunohistochimie (IHC) de tissus fixés dans la formaline et enrobés de paraffine. Dans 47 des 70 (67 %) tumeurs examinées, des lymphocytes T étaient en cause alors que dans 19/70 (27 %) il s'agissait de lymphocyte B et dans 4/70 (6 %) aucun des deux marqueurs n'a réagi avec les cellules. La mise en évidence du virus de la leucémie féline (VLFe) par IHC a été possible sur 38/70 tumeurs (54 %), et 52/70 tumeurs (74 %) étaient positives par réaction d'amplification en chaîne par la polymérase (ACP) pour la présence d'ADN du VLFe. Les tumeurs de type lymphocyte B étaient positives aussi fréquemment que celles de type lymphocyte T pour leur réactivité vis-à-vis la présence du VLFe tel que détectée par IHC ou ACP. Les tumeurs intestinales étaient plus fréquemment de type B que de type T. L'incidence des tumeurs de type B ou T ne différait pas selon qu'il s'agisse de jeunes chats (≤ 3 ans), de chats d'âge moyen (> 3 jusqu'à ≤ 8 ans) ou de vieux chats (> 8 ans). Indépendamment du type, les tumeurs rencontrés chez les vieux chats étaient VLFe positives plus souvent par ACP que par IHC. L'ADN, mais non l'antigène, du VLFe était détecté au niveau des tumeurs des lymphocytes de type B et des tumeurs intestinales aussi souvent chez les chats âgés de plus de 8 ans que chez ceux de 8 ans ou moins. Ce type de tumeurs chez les vieux chats étaient auparavant considérés comme étant négative pour la présence de VLFe. Les résultats de l'étude suggèrent l'implication de formes latentes ou ayant un défaut de réplication dans de telles tumeurs chez les vieux chats; et également que le VLFe joue un rôle dans le développement de tumeurs associées aussi bien aux lymphocytes B qu'aux lymphocytes T.

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INTRODUCTION

Lymphosarcoma (LSA) accounts for about 30% of all feline tumors, which is the highest rate of LSA in any outbred animal species (1–6). About 70% of feline LSAs are feline leukemia virus (FeLV)-related using antigen detection or virus isolation (7–9). However, we have reported (10) that FeLV DNA may be detected using the polymerase chain reaction (PCR) in additional LSAs particularly from older cats, suggesting that the virus may be associated with a larger proportion of LSAs than previously determined.

There are several reports characterizing the phenotype of feline LSAs in relation to anatomic distribution of the tumor, patient age, and/or FeLV status (6,11–15). Early studies related the anatomic distribution of neoplastic cells to specific compartments in

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lymphoid tissues, so that in alimentary LSA, the main tumor was described in Peyer's patches and germinal centers of mesenteric lymph nodes, suggesting B cell transformation. Whereas in thymic and multicentric LSA, the neoplastic cells occupied the paracortical or thymus-dependent areas, suggesting T cell transformation (16). Later studies involved T cell identification by nonimmune rosette formation of guinea pig erythrocytes (GPE), and B cell identification by demonstration of surface immunoglobulin (Ig) and/or demonstration of complement receptors by erythrocyte, antibody, complement (EAC) rosettes (11,13–15,17). In one report of 14 LSAs, thymic tumors were T cell, alimentary tumors were B cell, and multicentric tumors lacked B or T cell surface markers (11). In another study of 36 LSAs, all the thymic and just over half of the multicentric tumors were T cell, and most of the alimentary tumors were B cell (14). More recently, lymphocyte subtypes in 5 primary feline LSAs and 6 feline LSA cell lines were characterized using monoclonal and polyclonal antibodies, immunofluorescence, rosette-forming assays, and cytochemistry (12). Conclusions were that FeLV does not transform only mature T cells but also immature or prothymocytes, null cells, and possibly helper T cells or monocytes. Transformation of mature B cells was excluded by lack of surface Ig expression in the feline LSA cell lines and primary tumors that were examined (12). Most reports indicate that multicentric and mediastinal LSAs are T cell and FeLV-positive, while alimentary LSAs are B cell and mostly FeLV-negative (8,14,18,19). Non-producer LSA (FeLV antigen-negative) occurs most often in older cats and/or in cats with alimentary LSA (7,9), however, there is evidence to suggest FeLV involvement in at least a portion of non-producer LSA (7,10,20).

Much of the earlier work has been hindered by the fact that GPE rosette formation is not specific for T cells in the cat (21), limited numbers of spontaneous tumors were evaluated, and immunological markers recognizing T and B cells over a broad range of developmental stages and applicable to archival material, were not available. The development of the polymerase chain reaction (PCR) and immunohistochemistry (IHC) for detection of FeLV DNA (10,22,23) and antigen (10,24,25) respectively, in paraffin-embedded, formalin-fixed tissue, has facilitated retrospective study of FeLV-negative versus FeLV-positive but non-producer LSAs. Recently, a polyclonal rabbit anti-human CD3 antibody (Dako Corp., Carpinteria, California, USA) has been shown to detect T cells in formalin-fixed, paraffin-embedded feline lymphoid tissues (26). Also, a monoclonal antibody, RA3-6B2 (Cedarlane Laboratories Ltd., Hornby, Ontario) that recognizes only B lymphocytes, has been applied to formalin-fixed, paraffin-embedded feline lymphoid tissues and appears to stain B cells over a broad range of differentiation (27). The basic purpose of this study was to determine the phenotype of 70 formalin-fixed, paraffin-embedded feline LSAs using T and B cell-specific antibodies, and to relate lymphocyte type to FeLV status by IHC for antigen and PCR for viral DNA, anatomic form of the tumor, and patient age.

MATERIALS AND METHODS

Seventy LSA cases from the archives of the Pathology Department at the Western College of Veterinary Medicine, were investigated. Cats were grouped by age as follows, ≤ 3 y, > 3 y to ≤ 8 y, and > 8 y to represent young, middle-aged, and old cats. Lymphosarcomas were classified according to anatomic distribution of the major tumor lesions on gross necropsy (3,6). Multicentric LSA involved tumor in lymph nodes, spleen, liver, and kidneys, alone or in any combination; lymphocytic leukemia was considered a manifestation of multicentric LSA (2,28). Mediastinal LSA included tumors of the thymus and/or mediastinal lymph nodes. Alimentary LSA involved tumors at any site in the gastrointestinal tract and/or mesenteric lymph nodes (2).

The PCR targeted a 166 base pair segment of the FeLV U3 long terminal repeat (LTR) (22,23), and was carried out as previously described (10). Tissue controls and interpretation of PCR results were also as previously described (10). The avidin-biotin complex (ABC) method for automated IHC staining of formalin-fixed, paraffin-embedded tissues (29), was used for detection of FeLV, B cell, and T cell antigens. For immunohistochemical evaluation, duplicate sections of each block were tested for staining with each antigen. The FeLV envelope glycoprotein, gp70, was detected using 1:400 and 1:800 dilutions of a goat anti-FeLV gp70 primary antiserum (National Cancer Institute, Bethesda, Maryland, USA) with modifications as previously described (10). For B cell identification, RA3-6B2, a rat monoclonal antibody to mouse B cells (B220) (Cedarlane Laboratories Ltd, Hornby, Ontario), was used as described for application to formalin-fixed, paraffin-embedded feline tissues (27), at dilutions of 1:50 and 1:100. Antigen retrieval was enhanced using Target Unmasking Fluid (TUF) (Monosan, Uden, The Netherlands) and incubation in a microwave oven according to the manufacturer's instructions. T cells were identified using 1:400 and 1:800 dilutions of a polyclonal rabbit anti-human CD3 antibody (Dako corp., Carpinteria, California, USA). Negative controls were sections from each case in which the primary monoclonal antibody or polyclonal antiserum was sustituted with a similarly diluted species and isotype-matched irrelevant antibody or antiserum. All slides were examined and evaluated by technical staff alone, then together with one or more pathologists. Interpretation of lymphocyte staining was obvious for most slides. In lesions staining with both B and T cell markers, the identity of the neoplastic population was determined by consensus of 2 pathologists.

Tumor phenotype was compared among cats using the following characteristics: FeLV status, anatomic site, and patient age group. Also, FeLV infection rates, assessed using IHC and PCR, were compared using the following characteristics: location of B cell tumors (intestinal versus non-intestinal), age among cats with B cell LSAs, and age among cats with intestinal LSAs. These relationships were assessed using Pearson's chi-square test, or Fisher's exact test when appropriate (30). The odds ratios and 95% confidence intervals were assessed when the relationships were statistically significant ($P \leq$

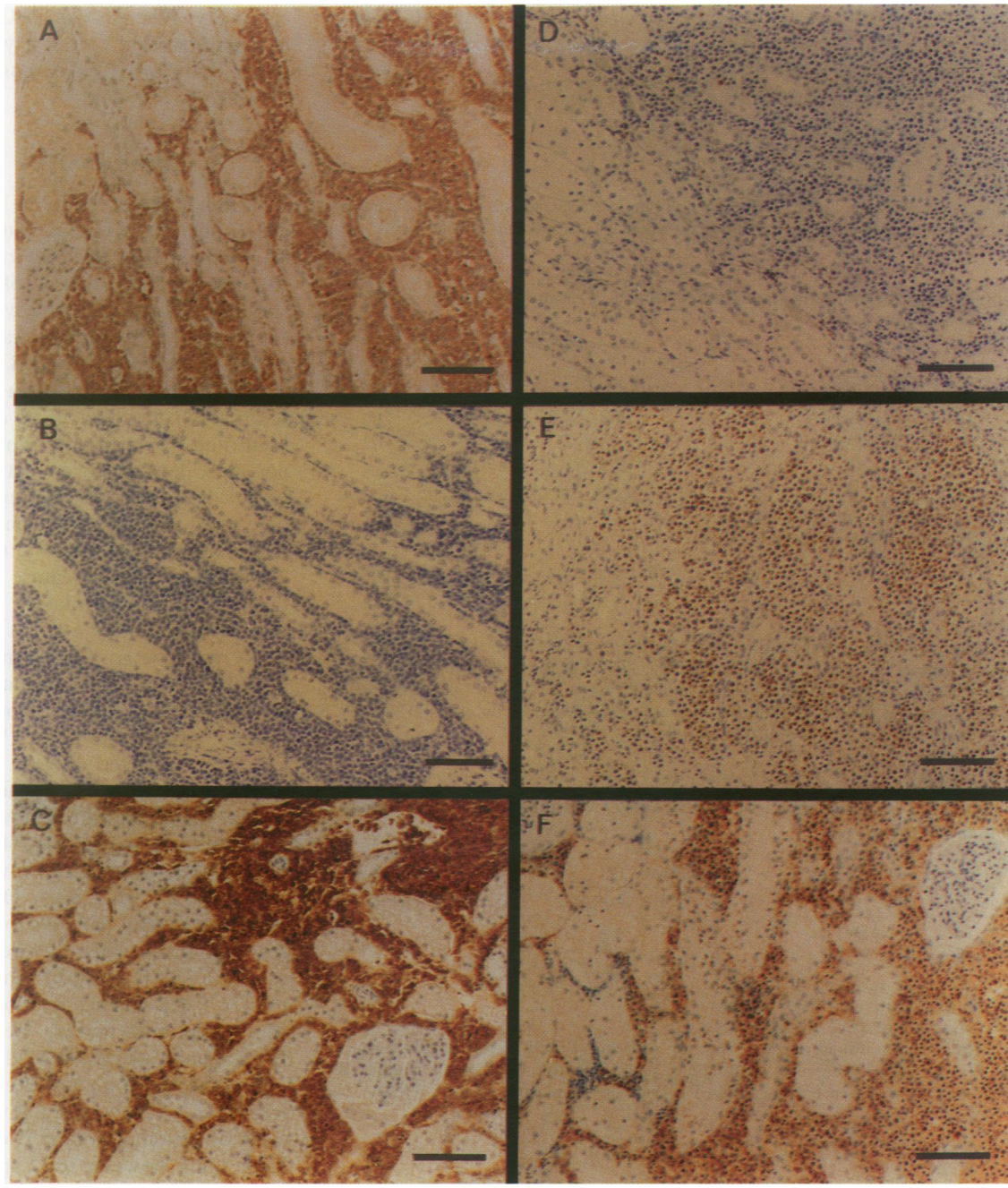

Figure 1. Formalin-fixed, paraffin-embedded kidney sections from 2 cats with lymphosarcoma. A, B, and C are from one cat, and D, E, and F from the other. Tissues have been stained with the T cell antibody in A and D, the B cell antibody in B and E, and the FeLV antibody in C and F. Dark brown staining indicates positivity for the T cell phenotype in A, the B cell phenotype in E, and FeLV antigen in C and F. Avidin-biotin complex immunoperoxidase with hematoxylin counterstain. Bar = 100 μm.

0.05) (31). Within tumor phenotype groups in cats ≥ 8 y, differences in FeLV detection rates using IHC and PCR were assessed using McNemar's chi-square test for two related samples (31). Tumors that were indeterminate for PCR results as previously defined (10), were included as PCR negative for the statistical calculations.

RESULTS

Positive and negative immunohistochemical staining using the T and B cell antibodies, and positive staining using the FeLV antibody, are shown for LSAs from 2 cats in Figure 1. Of 70 LSAs, 47 (67%) were T cell tumors, 19 (27%) were B cell tumors, and 4 (6%) did not stain with either T or B cell marker (Table I). Thirty-eight (54%) of 70 tumors were positive for FeLV antigen by IHC, and 52 (74%) were positive for FeLV DNA by PCR. The distribution of IHC-positive and PCR-positive tumors according to lymphocyte type is given in Table I. There was no difference in

TABLE I. Lymphocyte type and feline leukemia virus (FeLV) status of 70 feline lymphosarcomas

FeLV status	T cell (47)	B cell (19)	Not T/B cell (4)
IHC+[a] (38)	24	10	4
PCR+[b] (52)	34	14	4

[a] Immunohistochemistry positive for FeLV antigen
[b] Polymerase chain reaction positive for FeLV DNA
No significant difference was found in the ratio of T cell tumors that were FeLV positive compared to the ratio of B cell tumors that were FeLV positive by either IHC ($P = 0.91$) or PCR ($P = 0.91$)

TABLE II. Lymphocyte type and feline leukemia virus (FeLV) status of 70 feline lymphosarcomas according to anatomic site

	T cell (47)		B cell (19)		Not T/B cell (4)	
Site	*n*	IHC+[a]/PCR+[b]	*n*	IHC+/PCR+	*n*	IHC+/PCR+
Mul[c] (42)	27	17/23	12	7/9	3	3/3
Med[d] (10)	9	4/7	0		1	1/1
Int[e] (13)	6	2/2	7	3/5	0	
Cut[f] (5)	5	1/2	0		0	

[a] Immunohistochemistry positive for FeLV antigen
[b] Polymerase chain reaction positive for FeLV DNA
[c] Multicentric
[d] Mediastinal
[e] Intestinal
[f] Cutaneous
A significant difference was found in the ratio of B to T cell intestinal tumors compared to the ratio of B to T cell non-intestinal tumors (Fisher's exact 2-tailed, $P = 0.04$, odds ratio = 3.99, test-based 95% confidence interval = 0.96–17.07). No significant difference was found in FeLV detection between the B cell intestinal tumors and the B cell non-intestinal tumors by either IHC or PCR (Fisher's exact 2-tailed, $P = 0.53$ and $P = 0.65$, respectively)

TABLE III. Lymphocyte type and feline leukemia virus (FeLV) status of 70 feline lymphosarcomas according to age group

	T cell (47)		B cell (19)		Not T/B (4)	
Age group	*n*	IHC+[a]/PCR+[b]	*n*	IHC+/PCR+	*n*	IHC+/PCR+
≤ 3 y (26)	18	13/15	5	4/4	3	3/3
> 3–≤ 8 y (20)	13	9/13	6	5/6	1	1/1
> 8 y (24)	16	2/6	8	1/5	0	

[a] Immunohistochemistry positive for FeLV antigen
[b] Polymerase chain reaction positive for FeLV DNA
No significant difference was found in the proportion of T and B cell tumors according to age group ($P = 0.65$). A significant difference was found in FeLV detection by PCR compared to IHC in B cell tumors from cats > 8 y ($P = 0.045$), and in T cell tumors from cats > 8 y ($P = 0.02$)

the ratio of T cell LSAs that were FeLV-positive compared to the ratio of B cell LSAs that were FeLV-positive using either IHC or PCR. Table II shows the distribution of lymphocyte type and FeLV status according to anatomic site of the LSA. No mediastinal or cutaneous tumors were stained by the B cell antibody. Intestinal tumors were almost 4 times more likely to be B cell than T cell. Using either IHC or PCR, the ratio of FeLV-positive intestinal B cell tumors was not different from the ratio of FeLV-positive non-intestinal B cell tumors. Table III shows the distribution of lymphocyte type and FeLV status of the LSAs according to patient age group. The proportion of T to B cell tumors did not differ according to age group. The PCR detected FeLV DNA more often than IHC detected FeLV antigen in both B and T cell tumors from cats > 8 y. The distribution of lymphocyte type and FeLV status according to anatomic site of the LSA and patient age group is shown in Table IV. Using IHC, B cell tumors from cats ≤ 8 y were more likely to be FeLV-positive than B cell tumors from cats > 8 y. However using PCR, B cell tumors from cats ≤ 8 y were not more likely to be FeLV-positive than those from cats > 8 y. Similarly using IHC, intestinal tumors from cats ≤ 8y were more likely to be FeLV-positive than intestinal tumors from cats > 8 y. Whereas using PCR, intestinal tumors from cats ≤ 8 y were not more likely to be FeLV-positive than those from cats > 8 y.

DISCUSSION

In this study of 70 feline LSAs, the T cell phenotype predominated (67%). This agrees with the previous largest study which found that 23 of 36 (64%) spontaneous feline LSAs were T cell (14). However, we found that 19 of 70 (27%) of the tumors were B cell, compared to 4 of 36 (11%) in the same report (14). Previously, B cells were identified by surface Ig which would not allow detection of immature B cells (32), whereas the B cell marker used here facilitated identification of B cells over a broad developmental range (27). This suggests that immature B cells are targets for transformation by FeLV. Here, B cell tumors were as frequently FeLV-positive as T cell tumors using both IHC for FeLV antigen and PCR for FeLV DNA. B cell tumors are generally considered to be FeLV-negative (9,12,33), however, the lack of a B cell marker reactive over a broad developmental range, and a bias toward study of T cell tumors (12,13,18,34–38), may have precluded adequate investigation of feline B cell LSAs. One report describes several methods to characterize the transformed cell type in FeLV-related LSAs (12). Although the markers for T cell characterization were for immature and mature cell stages, B cells were identified by demonstrating surface Ig, a mature B cell characteristic (32). There was no evidence for transformation of mature B cells in the same study, however the 5 spontaneous tumors and 6 cell lines characterized were thymus and/or multicentric derived (12). In the present study, no mediastinal tumors and only 12 of 42 multicentric tumors were B cell so that only a subset of multicentric and intestinal tumors would demonstrate evidence of B cell transformation and these would not necessarily be mature B cells. Evidence for B cell transformation may not have been demonstrated in the previous study (12) because of the nature of the B cell markers used, or because of the select group of spontaneous tumors and cell lines investigated.

In the current study, there were no mediastinal or cutaneous B cell

tumors. Although there were more multicentric B cell tumors than intestinal B cell tumors, intestinal tumors were 4 times more likely to be B cell than T cell. This supports previous studies which indicate a preference for B cell tumors among those involving the intestinal tract (2,11,14,18,19,39), however, intestinal tumors were not exclusively B cell, nor were B cell tumors exclusively the intestinal form, in the present study. Intestinal B cell tumors were as likely to be FeLV-positive as non-intestinal B cell tumors (all non-intestinal tumors were multicentric tumors in this study) by both IHC and PCR. Also, there was no increased preference for B cell tumors in old cats. Previous findings that intestinal and/or B cell tumors are negative for FeLV (7,8,18,40), may relate more to the age of cat from which the tumor was derived than to lymphocyte type or anatomic form. Also, viral antigen rather than DNA was detected in most of the previous studies and we have demonstrated that FeLV DNA can be detected more often than FeLV antigen, particularly in older cats (10). In the current study, both B and T cell tumors from old cats were FeLV-positive by PCR more often than by IHC. Virus DNA-positive, antigen-negative tumors suggest infection with latent or replication-defective FeLV (10).

Here, FeLV DNA but not antigen, was detected in B cell and intestinal tumors from old cats as often as it was from young and middle-aged cats. However, DNA and antigen detection were not different when tumors were investigated by lymphocyte type and anatomic site. These findings provide additional support for viral latency or replication-defective forms in older cats rather than a lack of FeLV involvement in B cell and/or intestinal tumors.

This study supports a role for FeLV in feline B cell as well as T cell tumorigenesis. However, no attempt was made to determine if viral integration was clonal. In natural LSAs, non-clonal integration of FeLV suggests superinfection following clonal expansion, so the presence of FeLV would be considered coincidental to tumorigenesis (18). However, the likelihood of superinfection occurring in those B cell tumors from old cats with viral DNA but no viral antigen, is remote.

TABLE IV. Lymphocyte type and feline leukemia virus (FeLV) status of 70 feline lymphosarcomas according to anatomic site and age

Site and Age group	T cell (47)		B cell (19)		Not T/B cell (4)	
	n	IHC+[a]/PCR+[b]	*n*	IHC+/PCR+	*n*	IHC+/PCR+
Mul[c] (42)						
≤ 8 y	21	17/20	7	5/6	3	3/3
> 8 y	6	0/3	5	1/3	0	
Med[d] (10)						
≤ 8 y	8	4/6	0		1	1/1
> 8 y	1	0/1	0		0	
Int[e] (13)						
≤ 8 y	1	1/1	4	3/3	0	
> 8 y	5	1/1	3	0/2	0	
Cut[f] (5)						
≤ 8 y	1	0/1	0		0	
> 8 y	4	1/1	0		0	

[a] Immunohistochemistry positive for FeLV antigen
[b] Polymerase chain reaction positive for FeLV DNA
[c] Multicentric
[d] Mediastinal
[e] Intestinal
[f] Cutaneous

A significant difference was found in FeLV detection by IHC between B cell tumors from cats ≤ 8 y compared to B cell tumors from cats > 8 y (Fisher's exact 2-tailed, $P = 0.005$, odds ratio = 31, test-based 95% confidence interval = 1.72–1363.94). No significant difference was found in FeLV detection by PCR between B cell tumors from cats ≤ 8 y compared to B cell tumors from cats > 8 y (Fisher's exact 2-tailed, $P = 0.26$). A significant difference was found in FeLV detection by IHC between intestinal tumors from cats ≤ 8 y compared to intestinal tumors from cats > 8 y (Fisher's exact 2-tailed, $P = 0.03$, odds ratio = 28, test-based 95% confidence interval = 0.88–4883.46). No significant difference was found in FeLV detection by PCR between intestinal tumors from cats ≤ 8 y compared to intestinal tumors from cats > 8 y (Fisher's exact 2-tailed, $P = 0.27$)

The lymphocyte type of the 4 tumors that did not stain with either the B or the T cell marker, is not known. There may be subsets of B and/or T cells which are not detected by the antibodies used here, or these may represent null cell tumors. Monoclonal antibodies recognizing feline T cell subsets have been developed to study the distribution of lymphocyte subsets in normal feline tissues (41–44), and to examine lymphocyte subset alterations associated with FeLV and feline immunodeficiency virus (FIV) infections (32,45–47). However, these monoclonal antibodies have been applied mainly to flow cytometric analysis. Panels of markers for both B and T cell subsets and applicable to formalin-fixed, paraffin-embedded feline tissues would allow more detailed phenotyping of archival lymphoid tumors. As has been found with human lymphoid tumors (48,49), B and T cell subset identification of feline LSAs would allow evaluation of treatment protocols and prognostic factors in relation to specific tumor cell subtype. Determining the transformed B and T cell subsets in abundant archival feline LSAs and relating this to FeLV status, would contribute to our understanding of the pathogenesis of FeLV-induced versus FeLV-negative LSAs.

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