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MUM-1 in canine lymphoma: a pilot study.

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Keywords:	Diffuse large B-cell lymphoma, IRF4/MUM1 expression, Dog < Domestic Mammals < Species, Double immunohistochemistry, Peripheral T-cell lymphoma

Abstract:	Expression of interferon regulatory factor 4 (IRF4)/multiple myeloma oncogene-1 (MUM1) in peripheral T-cell lymphoma (PTCL) in people is associated with a poorer survival outcome compared to cases of PTCL lacking MUM1 expression. The aim of this study was to determine whether MUM1 is expressed in canine peripheral T-cell lymphoma not otherwise specified (PTCL-NOS). For comparison, the presence of MUM1 antigen was also investigated in canine diffuse large B cell lymphoma (DLBCL). Nine cases of PTCL-NOS and nine cases of DLBCL diagnosed by a commercial veterinary diagnostic laboratory were selected. Positive immunohistochemical labelling for MUM1 was observed in PTCL-NOS (2 out of 9 cases) and DLBCL (3 out of 9 cases). These findings suggest that a subset of neoplastic T and B lymphocytes can express MUM1. The role of MUM1 in the biological behaviour and outcome of canine lymphoma requires further investigation on a larger number of cases.

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1 **MUM-1 in canine lymphoma: a pilot study.**

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19 **Abstract**

20 Expression of interferon regulatory factor 4 (IRF4)/multiple myeloma oncogene-1
21 (MUM1) in peripheral T-cell lymphoma (PTCL) in people is associated with a poorer
22 survival outcome compared to cases of PTCL lacking MUM1 expression. The aim of
23 this study was to determine whether MUM1 is expressed in canine peripheral T-cell
24 lymphoma not otherwise specified (PTCL-NOS). For comparison, the presence of
25 MUM1 antigen was also investigated in canine diffuse large B cell lymphoma (DLBCL).
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28 for MUM1 was observed in PTCL-NOS (2 out of 9 cases) and DLBCL (3 out of 9 cases).
29 These findings suggest that a subset of neoplastic T and B lymphocytes can express
30 MUM1. The role of MUM1 in the biological behaviour and outcome of canine lymphoma
31 requires further investigation on a larger number of cases.

32 Keywords: diffuse large B-cell lymphoma, MUM1 expression, dog, double
33 immunohistochemistry, peripheral T-cell lymphoma

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35 Canine lymphoma (CL) is a common neoplasm with an estimated incidence rate of 20-
36 100 cases per 100,000 dogs and is comparable to non-Hodgkin lymphoma in humans.[20]
37 According to the World Health Organisation (WHO) classification criteria[16], CL has
38 different phenotypic subtypes. The subtype is known to have an impact on prognosis
39 and is used to inform treatment protocols.[9,20] Approximately 60-70% of CL are B-cell,
40 30-40% T-cell, and less than 1% null (non-B non-T cell).[11,17] Diffuse large B-cell
41 lymphoma (DLBCL) is the most common subtype of CL. Peripheral T-cell lymphoma not
42 otherwise specified (PTCL-NOS) is the second most common subtype of canine
43 lymphoma and comprises a heterogenous group of T-cell lymphomas. [11,17] High-grade
44 T-cell lymphoma is an aggressive disease, and when treated with cyclophosphamide,
45 hydroxydaunorubicin, vincristine, and prednisone (CHOP)-based protocols, dogs have a
46 complete remission rate as low as 40%, relapse earlier and have shorter survival time
47 than dogs with a comparable stage, high-grade B-cell lymphoma.[9]

48 Interferon regulatory factor 4 (IRF4)/multiple myeloma oncogene-1 (MUM1) is a
49 transcription factor associated with cell growth, particularly B-cell maturation. MUM1
50 immunohistochemistry is routinely used for diagnostic confirmation of plasma cell tumor
51 in dogs.[12] In addition, expression of MUM1 has been demonstrated in some canine
52 lymphomas of B-cell origin.[12] Expression of MUM1 within PTCL-NOS in humans is
53 associated with a poorer survival outcome compared to tumors which do not express
54 this transcription factor.[7,15] The purpose of this retrospective study is to determine
55 whether canine PTCLs-NOS express MUM1, and if the degree of expression differs
56 between DLBCLs and PTCLs-NOS.

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57 A retrospective search was performed at IDEXX Diagnostic Laboratories Ltd (Wetherby,
58 UK) on cases submitted between 2018 to 2020. Only cases with a diagnosis of PTCL-
59 NOS and DLBCL and the immunophenotype already confirmed by
60 immunohistochemistry for B and T cell antigens were included in this study. A total of 9
61 cases of PTCL-NOS and 9 cases of DLBCL were collected. The samples consisted of
62 whole node extirpation (17 samples) and Tru-cut biopsies (one case of PTCL-NOS).
63 Haematoxylin and eosin (H&E) stained slides and immunohistochemical slides of all
64 cases were reviewed by 2 board certified pathologists (ER, SLC) and classified
65 according to the WHO criteria.[16] The following features were recorded for each case:
66 nodular versus diffuse growth pattern, nuclear size and the number of mitoses per high-
67 power field. Nuclear size was determined as small (<1.5x the size of a red blood cell),
68 intermediate (1.5–2x the size of a red blood cell), or large (>2x the size of a red blood
69 cell). The number of mitotic figures was counted in one 40X digital high-power field
70 (HPF) in the area of highest mitotic activity and multiplied by 1.2 to calculate the mitotic
71 count (MC) in one standard HPF (0.237 mm^2) according to the Meuten et al.[8]
72 Lymphomas with 0 to 5 mitoses in 0.237 mm^2 were graded as low grade, those with 6 to
73 10 mitoses/0.237 mm^2 were graded as medium grade, and those with greater than 10
74 mitoses/0.237 mm^2 were graded as high grade.[16]
75 To demonstrate the presence of MUM1 antigen in the T-cell or B-cell population, a
76 double immunohistochemical procedure with the T cell marker CD3 or B cell marker
77 CD20 and MUM1 was performed according to the following method.[10] CD20 was
78 selected as B-cell marker for the double immunohistochemical procedure instead of

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79 PAX5 because CD20 labelling is membranous/cytoplasmic (instead of nuclear) and thus
80 did not interfere with the MUM1 labelling.

81 Briefly, three micrometers thick sections were cut and mounted on KP-PLUS positive
82 charged slides (Klinipath), incubated overnight at 37°C, deparaffinized in three changes
83 of Histo-Clear Histology Clearing Agent (National Diagnostics) and re-hydrated in
84 ethanol solutions of decreasing concentration.

85 For heat-induced epitope retrieval (HIER), sections were treated at full pressure with
86 Access Retrieval Unit (Menarini) in EDTA buffer (pH 9) for 90 seconds at 125°C. The
87 sections were then rinsed in Tris Tween buffer (pH 7.5).

88 Endogenous peroxidase activity was quenched at room temperature with 3% hydrogen
89 peroxide in phosphate buffered saline. The sections were incubated for 30 minutes at
90 room temperature with the primary antibody MUM1 (MUM1p clone, Dako) in a 1:50
91 dilution. The sections were then washed with Tris Tween buffer (pH 7.5) to remove the
92 excess primary antibody.

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93 For primary antibody detection, the sections were incubated with EnVision+ System
94 HRP labelled polymer anti-mouse secondary antibody (Dako) for 30 minutes at room
95 temperature. The sections were washed with Tris Tween buffer (pH 7.5) to remove any
96 excess labeled polymer and incubated with Envision FLEX HRP Magenta Substrate
97 Chromogen System (Dako) for 5 minutes. Excess Magenta was removed by washing
98 the section with distilled water followed by Tris Tween buffer (pH 7.5). Sections were
99 then treated with 200 mM sulphuric acid to remove residual HRP activity without
100 impacting upon the intensity of the HRP Magenta stain.

101 The sections were then treated for 5 minutes at room temperature with 3% hydrogen
102 peroxide in phosphate buffered saline (PBS) to quench endogenous peroxidase activity.
103 The sections were then incubated for 30 minutes with either CD3 antibody (polyclonal,
104 Dako) in a 1:300 dilution or CD20 antobody (polyclonal,Fisher), in a 1:700 dilution. For
105 primary antibody detection, the sections were incubated with EnVision+ System HRP
106 labelled polymer anti-rabbit secondary antibody (Dako) for 30 minutes at room
107 temperature.

108 Tris Tween buffer was used to remove excess labelled polymer from the sections. This
109 was followed by two 5-minute incubations with 3,3'-diaminobenzidine (DAB) substrate-
110 chromogen (EnVision+ System, Dako). Sections where rinsed twice with distilled water
111 for 5 minutes each time. Tissues were counterstained using Gill's hematoxylin and
112 mounted using clear resin and coverslips for long-term storage. A normal canine lymph
113 node was used as positive control; negative control was obtained relacing the primary
114 antibody with Dako REAL Antibody Diluent.

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115 A semi-quantitative evaluation of the double immunolabeled slides was performed
116 according to the criteria described by Heo et al [7]: cases with less than 5% neoplastic
117 cells showing positive immunolabelling for MUM1 antibody were considered negative,
118 while cases with more than 5% of neoplastic cells labelling positive were considered
119 positive.

120 To calculate the number of MUM1 positive neoplastic cells within each group of
121 lymphomas, digital image analysis was performed using an Aperio Leica CS2 scanner
122 with QuPath-Software (v.0.2.3). The complete area of the tissue was inspected, starting
123 from the left upper corner of the tissue, until 100 MUM1 positive cells were counted. The
124 ratio of MUM1 positive and CD3 (or CD20) positive and negative cells was determined.
125 A cell was considered double positive when it had both brown cytoplasmic and red
126 nuclear labelling.

127 The results of histological examination of PTCL-NOS cases are summarized in Table 1.
128 PTCL-NOS cases showed a diffuse growth pattern. Five out of 9 cases were composed
129 of intermediate-sized cells, 3 were composed of large cells, and one of small cells. The
130 histological grade was low in 4 cases, intermediate in 2, and high in 3 cases. None of
131 the PTCL-NOS showed plasmacytoid features.

132 DLBCL showed a diffuse growth pattern and large (6 out of 9 cases) or intermediate to
133 large (3 cases) cell size. The histological grade was intermediate in 5 cases and high in
134 4 cases. None of the DLBCL were subtyped as plasmablastic lymphoma.

135 MUM1labelling was nuclear in both PTCLs-NOS and DLBCLs.

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136 Semi-quantitative evaluation of the double immunolabelled slides detected the presence
137 of double positive cells in one out of 9 cases of PTCL-NOS (Fig. 1, a and b) and in 2 out
138 of 9 cases of DLBCL. With digital image analysis, this increased to 2 out of 9 cases of
139 PTCL-NOS and 3 out of 9 cases of DLBCL showing double positive cells. No difference
140 in the pattern and degree of MUM1-positive labelling was noted between PTCLs-NOS
141 and DLBCLs.

142 In humans, PTCLs are aggressive non-Hodgkin lymphomas characterized by frequent
143 relapse and poor survival outcome.[1,18] Most patients with PTCL are treated mainly with
144 anthracycline-based chemotherapies, such as CHOP (cyclophosphamide, doxorubicin,
145 vincristine, and prednisolone); however, their response to this therapy is not as good as
146 patients who have B-cell lymphomas.[3] MUM1 has been suggested as a potential
147 prognostic factor and therapeutic target in PTCL in humans.[5,7,15] In veterinary oncology,
148 the expression of MUM1 in canine lymphoma has previously been reported in a small
149 number of cases of B-cell lymphoma and anaplastic lymphoma.[12] To our knowledge, the
150 expression of MUM1 has not been previously investigated in canine PTCL-NOS.

151 In the present study, positive immunolabelling for MUM1 was demonstrated in canine
152 PTCL-NOS cells expressing CD3, using a double immunohistochemical assay. One of
153 the positive cases was a high-grade, large cell lymphoma. In human pathology, MUM1
154 is reported to be frequently expressed in anaplastic lymphoma of large cell type.[7,15,19] A
155 correlation between the cell size, grade, and MUM1 expression can be thus
156 hypothesized also in canine species; however, a study with a larger number of cases is
157 needed to evaluate this hypothesis.

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158 Based on the high variability in grade, cell size and MUM1 expression noted in PTCL-
159 NOS in this study series, it is likely that different subsets of neoplastic T lymphocytes
160 are grouped under the general definition of PTCL-NOS. Human DLBCL is also a
161 heterogeneous disease with a variable clinical course. The subclassification of DLBCL
162 according to the stages of B-cell differentiation during germinal centre maturation, has
163 prognostic significance. [2,13,14] For this reason, in human pathology,
164 immunohistochemical detection of MUM1 is used in conjunction with other markers to
165 determine the cell of origin of DLBCL.[4,6] A subset of canine B cell lymphomas also
166 express MUM1, as demonstrated by the results of this study and previously shown by
167 Ramos Vara et al. [12]

168 In conclusion, a better characterization of the expression profile of neoplastic cells in
169 canine PTCL-NOS and DLBCL, with the help of immunohistochemistry and/or gene
170 expression profiling, might allow an accurate subclassification and might also represent
171 an important part of the diagnostic work-up, helping with the development of new
172 therapeutic strategies. In addition, it might allow the identification of subtypes of
173 lymphoma that display a more favourable outcome and a better response to therapy.
174 Finally, prospective studies on a larger number of cases with complete follow-up data
175 are required to definitively establish a correlation among tumor grade, cell size, and
176 MUM1 expression and to determine any potential prognostic significance of MUM1 in
177 canine PTCL-NOS and DLBCL.

178

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238 **Figure legends**

239 Figure 1. **a.** PTCL-NOS, case no. 2, lymph node, dog. The neoplastic cells have strong
240 membranous immunolabelling for CD3. Nuclear immunolabelling for MUM1 is also
241 noted, diffusely, in the same neoplastic cells. **b.** PTCL-NOS, case no. 6, lymph node,
242 dog. The neoplastic cells have strong membranous immunolabelling for CD3. Nuclear
243 immunolabelling for MUM1 is also noted in 11% of the CD3-positive neoplastic cells.
244 Double immunohistochemistry for CD3 (DAB chromogen) and MUM1 (HRP Magenta
245 chromogen).

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Table 1. PTCL-NOS cases. Summary of the results of the histological analysis and evaluation of the double immunohistochemical labelling for CD3 and MUM1.

Case	Pattern	Cell size	Grade	Semi-quantitative[a]	Digital image analysis[b]
1	Diffuse	Large	Intermediate	-	0%
2	Diffuse	Large	High	+	100%
3	Diffuse	Small	Low	-	0%
4	Diffuse	Large	Low	-	0%
5	Diffuse	Intermediate	High	-	0%
6	n/a	Intermediate	Low	-	11%
7	Diffuse	Intermediate	High	-	0%
8	Diffuse	Intermediate	Low	-	0%
9	Diffuse	Intermediate	Intermediate	-	0%

[a] = according to the method described by Heo et al.; [b] = percentage of MUM1 positive neoplastic cells on 100 CD3 positive neoplastic cells. PTCL-NOS = peripheral T-cell lymphoma not otherwise specified; + = positive (> 5%); - = negative; n/a = not applicable (the growth pattern is not provided as only Tru-cut biopsies are submitted).

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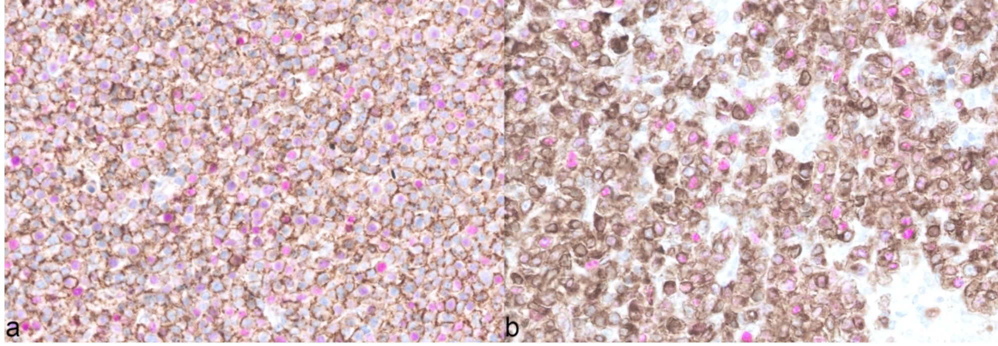

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