Positive Interplay Between CD3+ T-lymphocytes and Concurrent COX-2/EGFR Expression in Canine Malignant Mammary Tumors

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Abstract. *Background/Aim: The ability of tumors to evade the immune system is one of cancer hallmarks. In breast cancer, it has been demonstrated that the cyclooxygenase-2$^+$/ epidermal growth factor receptor$^+$ (COX-2$^+$/EGFR$^+$) status might influence tumor microenvironment allowing escape of cancer cells to the immune system. This topic is unknown in canine mammary tumors (CMT). Therefore, the potential relationship between CD3$^+$ T-lymphocytes and concurrent COX-2/EGFR expression was investigated. Materials and Methods: Formalin-fixed paraffin-embedded malignant CMT samples (n=63) were submitted to immunohistochemical staining to detect CD3, COX-2 and EGFR. Results: Tumoral CD3$^+$ T-lymphocytes were significantly associated with tubular differentiation grade (p=0.006), tumor necrosis (p=0.025), histological grade of malignancy (p=0.027) and presence of lymph node metastasis (p=0.009). A correlation between COX-2 and EGFR was observed (r=0.741, p<0.0001). The COX-2$^+$/EGFR$^+$ group was associated with tumor size (p=0.002), mitotic index (p=0.019), histological grade of malignancy (p=0.035) and presence of lymph node metastasis (p=0.041). CD3$^+$ T-lymphocytes and COX-2/EGFR groups were significantly associated (p=0.025) and positively correlated (r=0.399; p=0.003). Conclusion: The present results suggest that the COX-2$^+$/EGFR$^+$ status may be part of a strategy adopted by tumor cells to evade the cytotoxic tumor-specific immune responses.*

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Mammary tumorigenesis involves a complex and intricate interplay between tumor and stromal cells. The supportive tumoral microenvironment (fibroblasts, adipocytes and immune cells) surrounds primary tumor cells and appears to have a critical role in tumor progression towards malignancy (1-3).

In human breast cancer (4, 5) and in canine mammary tumors (CMT) (6-8), several studies have attributed an important role to CD3$^+$ T-lymphocytes, as well as cytokines produced by them. The evidence suggest that T-lymphocytes might cooperate with tumor cells favoring tumor development and progression (3, 4, 6-9).

Cyclooxigenase-2 (COX-2) over-expression has been related to tumor aggressiveness in human breast cancer (10, 11) and in CMT (12-16) and there has been a great interest in a better-understanding over the signaling pathways that underlie COX-2 expression. Several appointed mechanisms, which include de-regulated growth factor signaling and oncogene activation, have been reported (17). Examples of these mechanisms comprise activation of the Wnt pathway (18, 19) and the Ras-MAPK pathway (20) signaling *via* growth factor receptors, including epidermal growth factor receptor (EGFR) (21). COX-2 expression and prostaglandin

Table I. *Relationship between tumoral CD3+ T-lymphocytes and clinicopathological parameters.*

Clinicopathological parameters	Tumoral CD3			
	n	Mean	SE	*p*-Value
Tumor size				
T1 (<3cm)	20	92.81	22.69	
T2 (3-5cm)	19	59.80	6.87	0.111
T3 (≥5 cm)	24	122.20	23.80	
Skin ulceration				
Absent	46	89.47	13.19	0.301
Present	17	120.50	33.20	
Histological type				
"*In situ*" carcinoma	3	35.00	10.00	
Complex carcinoma	10	77.56	26.81	
Tubulopapillary carcinoma	32	103.72	10.00	0.210
Solid carcinoma	8	164.14	42.72	
Carcinosarcoma	7	52.00	9.24	
Anaplastic carcinoma	3	27.00	-	
Tumour necrosis				
Absent	36	72.83	11.15	0.025
Present	27	128.47	23.77	
Mitotic index				
1	28	67.62	10.37	
2	16	114.58	32.55	0.055
3	19	139.40	30.62	
Nuclear grade				
1	7	48.33	7.69	
2	28	105.62	19.74	0.622
3	28	100.37	20.11	
Differentiation grade				
1	14	58.40[a]	16.92	
2	24	69.00[a]	9.91	0.006
3	25	148.81[b]	26.62	
Histological grade of malignancy				
I	19	68.78[a]	10.67	
II	20	92.58[ab]	29.31	0.027
III	24	149.75[b]	29.82	
Lymph node metastasis				
Absent	28	72.74	11.64	0.009
Present	20	146.71	30.27	

n, Number of samples; SE, standard error; Mean values with different superscript letters denote statistically significant differences on each item considered-Tukey Post Hoc Test ($p<0.05$)

E2 (PGE2) production have been shown to up-regulate the EGFR, PI3K and ERK1/2 signaling, thereby inducing angiogenesis, cell proliferation and invasion (17, 22). COX-2 and EGFR molecules have been demonstrated to share some functions in common signaling pathways in several stages of mammary carcinogenesis by mediating pleiotropic carcinogenic processes both in humans and dogs (16, 23) .

In human breast cancer, COX-2 has an influence on tumor and stromal cell interplay and COX-2-derived PGE2 contributes to matrix remodeling, modulates multiple aspects of the immune responses and supports the suppressed immune surveillance (17, 24-26). PGE2 has the ability to regulate the immune system by modulating the functions of different cell populations, including T-lymphocytes (1, 26, 27), and has been reported to enhance pro-tumorigenic type-2 lymphocytes and myeloid cell functions promoting angiogenesis and supporting tumor growth (1, 27, 28).

COX-2 modulates and suppresses immune function in human breast cancer (28) and up-regulates the EGFR activity by a positive feedback loop in human and dog mammary tumors (16, 23), which raises the hypothesis that the ability of tumor cells to evade the immune system may, be influenced by inappropriate concurrent expression of COX-2/EGFR.

COX-2 and EGFR are promising therapeutic targets; therefore, the relationship between $CD3^+$ T-lymphocytes and concurrent COX-2/EGFR expression was investigated in the present study, since the better understanding over these molecular interplays may be useful in developing clinically effective immunotherapeutic approaches.

Materials and Methods

Tissue samples. In the present study, 63 malignant canine mammary tumors were included. Samples were surgically excised with curative intent from 63 dogs that expressed natural tumor occurrence. All specimens were fixed in 10% buffered formalin, paraffin-embedded and 2-μm sections were sequentially cut from each block, following routine methods. One section was stained with hematoxylin and eosin for histopathological diagnosis and subsequent sections were used for immunohistochemical studies. The histopathological diagnosis of tumors was performed by the WHO classification for CMT (29) by two independent pathologists (IP and JP). The clinicopathological characteristics, evaluated in each sample, were tumor size (T1 <3 cm; T2 ≥3 and <5 cm; T3 ≥5 cm), skin ulceration, presence of necrosis, mitotic index, nuclear grade, tubular differentiation grade, histological grade of malignancy and regional lymph node metastases. Mitotic index was assessed in 10 high-power fields (HPFs) (×400) and classified in 3 grades according to the recommended guidelines (30). Nuclear grade, tubular differentiation grade and histological grade of malignancy were also evaluated according to the recent recommendations for CMT grading (30).

Immunohistochemical analysis. Immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase complex method with the Ultra Vision Detection System kit (Lab Vision Corporation, Fremont, CA, USA) for CD3 and COX-2, while for EGFR was used a polymeric labeling methodology (Novolink Polymer Detection System; Novocastra, Newcastle, UK) following the manufacturer's instructions. Sections were de-waxed in xylene and rehydrated through graded alcohols. For CD3 and COX-2, antigen retrieval was executed by microwave treatment for 3×5 min at 750 W in 0.01 M citrate buffer, pH = 6.0, followed by cooling at room temperature for 20 min. For EGFR, antigen retrieval was carried out by enzyme digestion: sections were incubated with 0.4% pepsin (Dako, Glostrup, Denmark) in HCl 0.01 N solution (pH = 2) for 30 min at 37˚C. All sections were incubated with specific antibodies: CD3

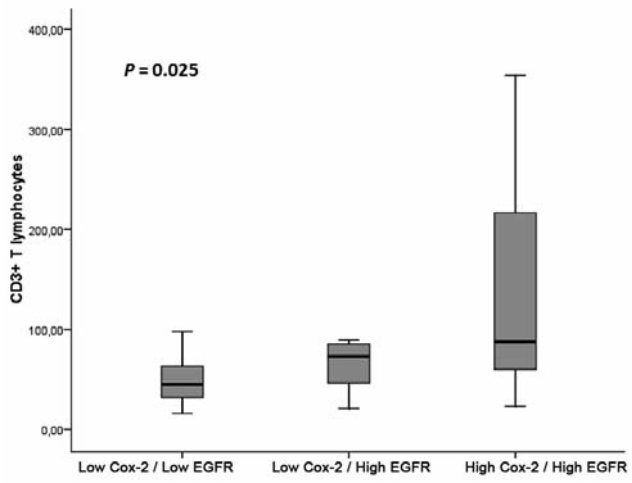

Figure 1. *Association of CD3+ T-lymphocytes and COX-2/EGFR groups in malignant canine mammary tumors.*

(polyclonal antibody; at 1:50 dilution; Dako, Glostrup, Denmark) for 2 h at room temperature; COX-2 (Clone SP21; at 1:40 dilution, Transduction Laboratories, Lexington, Kentucky, USA) for 24 h at 4˚C; EGFR (clone 31G7; at 1:100 dilution; Invitrogen, Paisley, Scotland, UK) for 45 min at room temperature. The antibody reaction products were observed with the cromagen 3, 3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). After a final washing in distilled water, the sections were counterstained with hematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with phosphate-buffered saline (PBS) for negative controls; this study also included adequate positive controls. Sections of canine lymph nodes were used as positive control for CD3. For COX-2, macula densa of young dog kidney was used, while the epidermis was used as internal positive control for EGFR.

Quantification of immunolabeling. The immunolabeling quantification was done by two independent observers (MIC and FLQ). To evaluate intratumoral CD3 expression, the three regions in the tumor with the most intense and homogeneous positivity were selected. In these regions, all labeled cells were counted, evaluating a total of 10 high power fields (HPFs) (×400) following a quantitative method used previously by our team (6). To evaluate COX-2 and EGFR expression, a previously applied semiquantitative method (16, 31) adapted from Ceccarelli and colleagues (32) was used. This method was based on the estimates of the percentage of positive cells (immunolabeling extension) and the staining intensity.

Statistical analysis. The statistical software SPSS (Statistical Package for the Social Sciences), version 19.0 (IBM SPSS Statistics), was used for statistical analysis. The Chi-square test was used to study the categorical variables. Analysis of variance (ANOVA) was used for analyzing continuous variables. The Pearson's correlation test was performed in order to verify the presence of correlation between values of CD3, COX-2 and EGFR. All values were expressed as means±standard error. In all statistical comparisons, $p<0.05$ was regarded as significant.

Results

Tumors. The present study comprised of 63 malignant canine mammary tumors, including 3 "*in situ*" carcinomas (4.8%), 10 complex carcinomas (15.9%), 32 tubulopapillary carcinomas (50.7%), 8 solid carcinomas (12.7%), 7 carcinosarcomas (11.1%) and 3 anaplastic carcinomas (4.8%). Nineteen malignant tumors were grade I, 20 grade II and 24 grade III. Within the 48 cases, where lymph nodes were available, 20 (41.67%) had metastases.

CD3+ T-lymphocytes, COX-2 and EGFR immunostaining. CD3+ T-lymphocyte were present in all samples ranging from 16 to 356 lymphocytes in 10 HPFs. CD3 immunostaining was observed in the cytoplasm and/or in the cytoplasmatic membrane of T-lymphocytes in a diffuse and homogeneous pattern. T-lymphocytes tend to contact closely with neoplastic cells and the diffuse inflammation emerged as the predominant pattern of infiltration. Sometimes, although less frequent, T-lymphocytes were also accumulated in perilobular and perivascular clusters.

Immunostaining for COX-2 and EGFR was also performed in all cases. The immunoreactivity for COX-2 was observed in the cytoplasm, nuclear membrane and cytoplasmatic membrane, in a diffuse and homogeneous manner. Thirty one of the 63 cases demonstrated high immunoreactivity for COX-2. The immunoreactivity for EGFR was observed at the cytoplasmatic membrane and within the cytoplasm of the neoplastic cells, in a diffuse pattern. Thirty nine of the 63 cases showed high immunoreactivity for EGFR.

Relationship of CD3+ T-lymphocytes with clinico-pathological variables. The present results demonstrated an association between tumoral CD3+ T-lymphocytes and the tubular differentiation grade ($p=0.006$) showing that poorly differentiated tumors (with less tubular formation) demonstrated increased CD3+ infiltration. An association was also observed between increased CD3+ infiltration and presence of tumor necrosis ($p=0.025$), high histological grade of malignancy ($p=0.027$) and presence of lymph node metastasis ($p=0.009$). All results are summarized in Table I.

Correlation between COX-2 and EGFR and relationship of COX-2/EGFR groups with clinicopathologic variables. A positive and statistically significant correlation between COX-2 and EGFR immunoreactivity was observed ($r=0.741$, $p<0.0001$) and there were not any tumors with elevated COX-2 and low EGFR expression. Thirty one out of the 63 tumors (49.2%) with high COX-2 immunoreactivity had also high EGFR immunostaining. In this study, the COX-2/EGFR groups were considered: low COX-2/low EGFR (n=24); low COX-2/high EGFR (n=8); high COX-2/high EGFR (n=31).

Table II. *Relationship of COX-2/EGFR groups with clinicopathologic variables.*

Clinicopathological parameters	Low COX-2/low EGFR *n*	Low COX-2/high EGFR *n*	High COX-2/high EGFR *n*	*p*-Values
Tumor size				
1 (<3cm)	13	2	5	
2 (3-5cm)	8	3	8	0.002
3 (>5cm)	3	3	18	
Skin ulceration				
Absent	17	7	22	
Present	7	1	9	0.375
Histological type				
"*In situ*" carcinoma	1	0	2	
Complex carcinoma	5	1	4	
Tubulopapillary carcinoma	14	3	15	
Solid carcinoma	1	0	7	
Carcinosarcoma	3	1	3	0.251
Anaplastic carcinoma	0	3	0	
Tumor necrosis				
Absent	17	6	13	0.089
Present	7	2	18	
Mitotic index				
1	16	3	9	
2	4	2	10	0.019
3	4	3	12	
Nuclear grade				
1	5	0	2	
2	10	3	15	0.698
3	9	5	14	
Differentiation grade				
1	6	1	7	
2	11	4	9	0.340
3	7	3	15	
6	1	7		
Histological grade of malignancy				
I	10	2	7	
II	8	4	8	0.035
III	6	2	16	
Lymph node metastasis				
Absent	11	4	13	0.041
Present	5	3	12	

n, Number of samples;

The COX-2/EGFR groups were statistically significantly associated with tumor size (p=0.002), mitotic index (p=0.019), histological grade of malignancy (p=0.035) and presence of lymph node metastasis (p=0.041). More information is provided in Table II.

$CD3^+$ T-lymphocytes and COX-2/EGFR groups associations. A significant association between $CD3^+$ T-lymphocytes and COX-2/EGFR groups was observed (p=0.025). The group with high COX-2 and high EGFR demonstrated higher counts of tumoral $CD3^+$ T-lymphocytes (Figure 1).

Correlation between $CD3^+$ T-lymphocytes and COX-2/EGFR groups. In the present study, a positive and statistically significant correlation between $CD3^+$ T-lymphocytes and COX-2/EGFR groups was observed (r=0.399; p=0.003).

Discussion

Tumor-associated T-lymphocyte responses can be generalized to type 1 and type 2, in which Th1 lymphocytes limit tumor development and Th2 lymphocytes favor immune escape and disease progression. Both human and dog cancer patients seem to demonstrate a lymphocyte dysfunction characterized by an imbalance of the normal ratio of Th1/Th2 cells (1, 9, 33-35).

In human breast cancer, recent findings suggests that COX-2 and COX-2-derived products, particularly PGE2, act in tumor cells *via* classical cancer signaling pathways promoting tumorigenesis and playing critical roles in T cell responses, suppressing cytotoxic T cell actions against the tumor (26). PGE2 has been also reported to enhance protumorigenic type 2 lymphocytes (28) and to up-regulate the EGFR *via* by a positive feedback loop (23). COX-2/EGFR up-regulated pathways have been described as a major determinant for breast cancer progression and metastasis largely due to the ability to regulate and suppress the cytotoxic responses of the immune system (17, 26, 36, 37). In CMT, this topic remains unclear and, to the best of our knowledge, this is the first study to investigate the relationship between $CD3^+$ T-lymphocytes and concurrent COX-2/EGFR immunoexpression.

The present study revealed a relationship between high tumoral $CD3^+$ T-lymphocytes and presence of tumor necrosis, high differentiation grade, high histological grade of malignancy and presence of lymph node metastases. These results suggest an association of $CD3^+$ T-lymphocytes and more aggressive tumor phenotypes reflecting the involvement of T-lymphocytes in canine mammary malignancy. Our results are in agreement with previous published works in CMT (6-8, 38, 39) and suggest that the immune system may release factors that contribute to tumor survival, growth and invasion. Tumor cells might, thus, use a multitude of mechanisms to escape from cytotoxic T-cell actions and additionally be subject to the polarity of the pro-tumorigenic Th2 cell responses, which also work favoring tumor protection (9).

Concerning the concurrent COX-2/EGFR immunoexpression, the present results revealed a positive and statistically significant correlation between the two markers. Tumors with high COX-2 and EGFR immunoexpression were statistically associated with larger tumor size, high mitotic index, high histological grade of malignancy and presence of lymph node metastases. These results were already observed by our team in a small set of tumors (16) and are in agreement with studies in human cancer confirming the common aspects of the interactive signaling pathways between COX-2 and EGFR in both species (16, 37). Considerable evidence indicates that COX-2–derived PGE2 can activate EGFR signaling and, thereby, stimulate tumor cell proliferation, invasion and metastases (23).

Interestingly, in the current study, the concurrent COX-2/EGFR-positive expression was significantly associated with higher tumoral $CD3^+$ T-lymphocytes. Furthermore, a positive and statistically significant correlation was observed. According to the present results, tumoral $CD3^+$ T-lymphocytes may be influenced by inappropriate expression of COX-2/EGFR. COX-2 over-expression and the resulting increase in PGE2 levels could induce over-expression of EGFR, possibly representing a strategy adopted by tumors that contributes to the evasion of tumor-specific immune response. PGE2 induces suppression of antigen-presenting dendritic cells leading to a reduced activation of anti-tumor cytotoxic $CD8^+$ T-cells (24, 40). PGE2 also has inhibitory effects on T-cell apoptosis and decreases production of interferon gamma (IFNγ) and interleukin-2 (IL-2) (27, 41, 42). The cellular effects of PGE2 are mediated through four prostaglandin E receptors, EP1, EP2, EP3 and EP4 that are associated to different intracellular signaling pathways (43). Proliferation of Th1 cells is inhibited through EP2 (44). The EP2 and, maybe, the EP4 receptors mediated the suppressive effects of PGE2 on cytotoxic T cells (45).

The results of our work suggest that similar mechanisms may be present in CMT. The interaction between COX-2/EGFR and $CD3^+$ T-lymphocytes highlights the molecular connection between cancer therapy and cancer prevention and the growing importance of molecular targeted approaches. However, the mechanisms through which COX-2/EGFR influence the T-lymphocyte functions are still poorly-defined emphasizing the need for additional studies in this area.

Conclusion

The findings of our study support future investigations concerning the better understanding over the crosstalk between COX-2/EGFR signaling pathways and $CD3^+$ T-lymphocytes. The significant correlation of COX-2/EGFR with $CD3^+$ T-lymphocytes and the relationship of the molecular markers with more aggressive tumor phenotypes justify the need to pursue further studies considering clinically effective immunotherapeutic approaches against CMT.

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Conflicts of Interest

The Authors declare they have no competing interests.

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