Standardized approach for evaluating tumor infiltrating lymphocytes in canine mammary carcinoma: Spatial distribution and score as relevant features of tumor malignancy

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Neoplastic cells, through immunoediting mechanisms, can establish a state of immunosuppression to evade host immune defenses. The aims of this study were: (1) to validate a standard method for assessing tumor infiltrating lymphocytes (TILs) in canine mammary carcinoma by applying international human breast cancer guidelines; (2) to investigate if the TILs population was composed of a subset of regulatory T lymphocytes (Tregs); and (3) to evaluate the relationship between the number of TILs and Tregs and the biological behavior of the tumors. One hundred and twenty-nine canine mammary tumors were retrospectively selected for this study. Histological diagnosis, grading and histological evaluation of TILs was performed on hematoxylin and eosin-stained sections. TILs were evaluated using a three-tier semiquantitative method, previously validated in human medicine, based on the percentage of TILs (0–10%, 11–40% and 41–90%). Lymphocyte immunophenotype was confirmed by CD3 and CD79, while an anti-FoxP3 antibody was used to determine the presence of Tregs. The number of stromal TILs and invasive front TILs significantly correlated with each other ($P < 0.0001$) and increased with increasing histological grade ($P = 0.002$ and $P = 0.004$, respectively). A subset of TILs was composed of $FOXP3^+$ Tregs. Stromal Tregs and invasive front Tregs were associated with stromal TILs and invasive front TILs ($P = 0.03$; $P = 0.01$ and $P = 0.003$; $P = 0.007$, respectively). In conclusion, in canine mammary carcinomas, an increased number of stromal and invasive front TILs is associated with increased malignancy and significant increase of Tregs that could lead to immunosuppression and evasion of the host immune system.

Keywords

Canine mammary carcinoma, $FOXP3^+$ T regulatory lymphocytes, Immunohistochemistry, Immunosuppression, Tumor infiltrating lymphocytes

Introduction

Cancer immunoediting is the ability of a tumor to determine immunotolerance and immunosuppression (Hanahan et al., 2011). Cancer immunoediting is the process by which the immune system may constrain or promote tumor development and it occurs through three canonical phases: elimination; equilibrium and escape (O'Donnel et al., 2019). The first step, the elimination phase, is characterized by cooperation of the innate and adaptive immune response to eliminate transformed cells that are still not clinically visible as a tumor (O'Donnel et al., 2019). If transformed cells survive the elimination phase, the cancer progresses to the equilibrium phase in which growth is limited and the adaptive immune response edits cellular immunogenicity (O'Donnel et al., 2019). Edited tumors can proceed to the escape phase in which growth is unopposed via activation of immunosuppressive and immunoevasive pathways.

Immunotolerance is based on the selective outgrowth of antigenic variants with reduced immunogenicity (Kumar, 2021) that the host immune system does not recognize as non-self. Immune evasion can occur through the loss or reduced expression of MHC molecules by tumor cells, which prevents attack by cytotoxic T cells (Kumar, 2021). Immune checkpoint activation is a recognized immunoregulatory mechanism and the target of promising cancer immunotherapy. Tumor-mediated immune checkpoint activation includes stimulation of the inhibitory receptor CTLA-4 on effector T cells and upregulation of cell surface proteins that activate the programmed death-1 (PD-1) receptor on effector T cells (PD-L1 and PD-L2). Immunosuppression can also occur when a tumor secretes immunosuppressive factors such as TGF-β, interleukin 10, interleukin 35, prostaglandin E2, granzymes and galectins (Veiga-Parga, 2016; Kumar, 2021).

Regulatory T lymphocytes (Tregs) are a subtype of T lymphocytes which are essential in controlling the immune response (Lucca and Dominguez-Villar, 2020). The number of Tregs decreases in autoimmune disease but increases in cancer, promoting protumor activity (Lucca and Dominguez-Villar, 2020). Tregs are recruited by neoplastic cells to the tumor site where they inhibit the activity of anti-tumor immune cells. The immunosuppressive action of Tregs is supported by T helper 2 lymphocytes, M2 macrophages, type 2 dendritic cells and myeloid-derived suppressors. This results in inactivation of cytotoxic T lymphocytes, natural killer cells, T helper 1 lymphocytes, M1 macrophages and type 1 dendritic cells, which allows neoplastic cells to replicate, invade tumor stroma and eventually give rise to lymph nodeand systemic metastases (Salgado et al., 2015).

Tumor infiltrating lymphocytes (TILs) significantly influence the response to therapy in human breast cancer. Furthermore, a Treg-rich inflammatory microenvironment can inhibit the action of chemotherapy (Lee et al., 2013). TILs have been previously investigated in caninemammary tumors (Carvalho et al., 2011, Saeki et al., 2012, Kim et al., 2013, Franzoni et al., 2019), but different evaluation systems were used across the studies. Since a standardized method for TILs evaluation is lacking, the aims of this study were: (1) to validate a method for TILs evaluation in canine mammary carcinoma by applying international guidelines for TILs in human breast cancer; (2) to investigate whether the TILs populationwas composed of a subset of Treg lymphocytes; and (3) to evaluate the relationship between TILs and the number of Treg lymphocytes and the biological behavior of canine mammary carcinoma.

2. Materials and methods

2.1. Case collection, histological diagnosis and grading

Formalin-fixed paraffin-embedded (FFPE) canine mammary carcinomasamples collected between 2016 and 2019 were retrospectively selected from the database of the Pathology Service of the Department of Veterinary Medical Science, University of Bologna. Selected cases included neoplasms obtained from nodulectomy, regional or radical mastectomy. Incisional biopsies were excluded. Sections of 4 μm thick were cut from FFPE samples and routinely stained with hematoxylin and eosin (H-E). Tumor diagnoses were revised according to the current histological classification of canine mammary tumors (Zappulli et al., 2019). Tumors were graded based on the canine-adapted Nottingham system (Peña et al., 2012). If radical or regional inguinal mastectomy was performed, lymph nodes were always evaluated.

2.2. TIL evaluation

Stromal TILs were defined as the percentage of TILs present in the intra-tumoral stroma between cancer cells but not infiltrating tumor cell nests (Fig. 1A). Invasive front TILs were defined as TILs in the immediate peritumoral area of the stroma at the invasive front (Fig. 1B). Peripheral TILs were defined as TILs in the stroma of adjacent mammary lobules (Fig. 1C).

TILs were assessed on H-E sections using a modified system based on the guidelines for human breast cancer proposed by the International TILs Working Group (Salgado et al., 2015), in which only stromal TILs are considered prognostically relevant (Salgado et al., 2015). In order to stratify the spatial distribution of TILs in dogs, we evaluated TILs at the invasive front and in the peripheral mammary gland, in addition to stromal TILs.

The cases were evaluated by two pathologists (LVM, GS). Whole sections were first examined at low power (4x) to observe the entire tumor stroma, the invasive tumor front and the presence of adjacent non-neoplastic normal or hyperplastic mammary gland.

TILs included lymphocytes and plasma cells but excluded polymorphonuclear cells and macrophages. As indicated in the 2015 guidelines proposed by Salgado et al., TILs scoring was performed by examining entire sections and not only areas with the highest TILs density (hot spots/tertiary lymphoid structures). For tumors with heterogenous TILs content, a mean value was estimated based on evaluation of entire sections. Necrotic areas were avoided. The percentages of TILs were semi-quantitatively evaluated at 20x and reported as the percentage of tissue occupied by TILs within the total stromal area. Based on these results, stromal TILs, invasive front TILs and peripheral TILs were parsed into the following three categories (Fig. 2): low, 0–10% TILs; intermediate, 11–40% TILs; and high, 41–90% TILs. For the 'high' category, the maximum value was 90% because, as reported by Salgado and colleagues (Salgado et al., 2015), lymphocytes usually do not form solid cellular aggregates. Instead, there is generally some empty tissue space between individual lymphocytes and thus lymphocytes never occupy 100% of an area.

2.3. Immunohistochemistry

Three μm thick sections were dewaxed in xylene and rehydrated.
Endogenous peroxidase was blocked by immersion in 3% H_2O_2 diluted in methanol for 30 min. Antigen retrieval was performed for FOXP3 by incubation in a pH 6.0 citrate buffer heated for 20 min in a microwave oven at 750 W. Antigen retrieval for CD3 and CD79 was performed by incubation in a pH 8.0 EDTA buffer heated for 10 min in a microwave oven at 750 W. Slides were then incubated for 30 min in a blocking solution containing 10% normal goat serum in PBS. Sections were incubated overnight at 4 °C with antibodies against FOXP3 (clone FJK-16 s, Invitrogen, 1:400), CD3 (clone CD3–12, Leucocyte's antigen laboratory, UC Davis, 1:40) and CD79 (clone HM57, Santa Cruz, 1:400). Antibody binding was visualized using a commercial avidin-biotin peroxidase kit (VECTASTAIN ABC Kits, Peterborough, UK). The chromogen DAB (3,30 diaminobenzidine) was used. Slides

were counterstained with Harris's haematoxylin. For the negative controls, the primary antibody was replaced with an irrelevant, isotype-matched antibody to control for nonspecific binding of the secondary antibody. Normal canine lymph nodes were used as positive controls.

CD3 and CD79 immunostaining were used to identify the presence of T and B lymphocytes which was semiquantitatively evaluated as: T cell-prevalent; B cell-prevalent or T and B cells in similar amounts.

2.4. T reg quantification

For each tumor, the number of $FOXP3^+$ lymphocytes was obtained by calculating the mean count across five high power field images (310477 μm^2 per image) captured with a Nikon Eclipse E600 ocular field number of 22 (Nikon Corporation, Tokyo, Japan). The count was manually performed using a software.[1] After assessing the normality of the data, the median FOXP3 value was calculated for each tumor and used as a cut off to divide the cases into two categories: FOXP3 low- or high-infiltration groups (Papaioannou et al., 2019). Counts were performed in the stroma (stromal FOXP3) and in the invasive front (invasive front FOXP3).

2.5. Follow-up

In this study, 48-month clinical follow-up data were retrospectively collected. Tumor recurrence, lymph node and systemic metastases and survival status were assessed. Clinico-pathological information relating to follow-up was obtained via telephone interview with veterinary practitioners or owners and was used to define the overall survival (OS), tumor-specific survival (TSS) and disease-free survival (DFS). OS was calculated as the number of days between histological diagnosis and all-cause death of dogs studied. TSS was calculated as the number of days between diagnosis and death because of the

canine mammary carcinoma. DFS was defined as the number of days between histological diagnosis and local recurrence of the neoplasm in the same anatomical site (Rasotto et al., 2017, Nguyen et al., 2018).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism software Version 9 (GraphPad Software, San Diego, CA, USA). Normality was assessed by the D′Agostino-Pearson omnibus test. Categorical data were analyzed using the Chi square test. The Log-Rank test was used to evaluate the survival variables. *P* values < 0.05 were considered significant.

3. Results

3.1. Case series, histological diagnosis, and grading

This study assessed 129 mammary carcinomas from 122 dogs, including 75 intact females, 40 spayed females and seven animals for which sex data was not available. The mean age was 9.5 years (range 2–15 years). Median tumor size was 18 mm (range 3–95 mm). Histological diagnoses included 28 complex carcinomas, 19 mixed carcinomas, 16 solid carcinomas, 15 simple tubulopapillary carcinomas, 14 intraductal papillary carcinomas, eight simple tubular carcinomas, eight comedocarcinomas, six ductal carcinomas, four carcinomas arising in complex adenoma, three invasive micropapillary carcinomas, two anaplastic carcinomas, one spindle cell carcinoma, one adenosquamous carcinoma, one carcinoma and malignant myoepithelioma, one inflammatory carcinoma and one papillary carcinoma of the nipple. Tumours were graded as grade I in 46 cases (36%), grade II in 51 cases (39%) and grade III in 32 cases (25%).

3.2. Follow-up

Follow-up data were obtained for 57 of the 122 dogs. The inguinal lymph node contained metastases in three of the 38 cases for which the inguinal lymph node was available for histological analysis. Systemic metastases diagnosed with imaging were reported in five dogs. Twenty-five (44%) of the 57 dogs with follow-up data died (including five that died from tumor-related causes) and 32 (56%) were alive at the end of the study. The median OS was 990 days (range 30–2220 days) and the median TSS was 130 days (range 30–1080 days). DFS was available in 31 dogs, with a median of 1005 days (range 30–2220 days). Recurrence occurred in four of 31 dogs.

3.3. TILs scoring and spatial distribution, and their associations with clinicopathological findings

TILs were present in the tumor stroma, at the invasion front and at the periphery. There were more tumors with low numbers of stromal, invasive front and peripheral TILs than with intermediate or high TILs infiltration ($P < 0.0001$, Table 1). Invasive front TILs and peripheral TILs were not assessable in seven and 26 samples, respectively. The number of stromal TILs and invasive front TILs were significantly associated ($P < 0.0001$).

The distribution of carcinomas according to grade (I, II, III), TILs scoring (low, intermediate and high) and spatial areas (stromal, invasive front and peripheral) are summarized in Table 2. The number of stromal TILs and invasive front TILs were both significantly associated with the histological grade of the tumor ($P = 0.002$ and $P = 0.004$, respectively, Fig. 3). Grade I carcinomas were characterized by a mild TILs infiltration (low group), while grade III carcinomas were associated with a marked TILs infiltration (high group).

An increase in stromal TILs was significantly associated with the presence of lymph node metastases ($P = 0.013$). However, TILs, both stromal and at the invasive front, were not

associated with OS ($P = 0.71$ and 0.11, respectively), TSS ($P = 0.92$ and 0.13, respectively) or DFS ($P = 0.53$ and 0.45, respectively). Statistical data are summarized in Table 3.

3.4. TILs immunophenotype

TILs were confirmed to be T (CD3-positive) or B (CD79-positive) lymphocytes. Semiquantitative TIL evaluation confirmed the presence of a mixed population of lymphocytes with a clear prevalence of $CD3^+$ cells in the majority of tumors (in the stromal areas in 79% of cases, at the invasive front in 66% of cases and in the adjacent normal mammary gland in 59% of cases). An equal prevalence of $CD3^+$ and $CD79^+$ lymphocytes was observed in the stromal areas in 21% of cases, at the invasive front in 32% of cases and in the adjacent normal mammary gland in 41% of cases. In contrast, a prevalence of CD79 + lymphocytes were very rare (at the invasive front in 2% of cases and not observed in the stroma or in the normal mammary gland). An example of the relative proportion of B and T lymphocytes is shown in Supplementary Fig. 1 (see Appendix A: Supplementary material).

A subset of TILs both in the tumor stroma and at the invasive front showed intensely positive nuclear immunostaining for FOXP3 and therefore, were identified as Tregs. The median values of $FOXP3^+$ stromal lymphocytes (stromal Tregs) and $FoxP3^+$ lymphocytes on the invasive front (invasive front Tregs) were calculated (stromal TILs, median = 9, range 0–221.7; invasive front TILs, median = 9, range 0–168) and used as a cut-off to separate cases into high and low FOXP3-infiltration groups. Sixty-one carcinomas had a low number of stromal $FOXP3^+$ cells (Fig. 4A) and 62 carcinomas had a high number of stromal $FOXP3^+$ cells (Fig. 4B).

The number of invasive front Tregs were significantly and positively associated with the number of stromal Tregs ($P = 0.008$). The number of stromal Tregs and invasive front Tregs were significantly associated and with the number of stromal TILs and invasive front

TILs ($P = 0.03$; $P = 0.01$ and $P = 0.003$; $P = 0.007$, respectively). In fact, carcinomas infiltrated by a low number of stromal TILs and invasive front TILs also had a lower than median number (< 9) of invasive front Tregs. Conversely, carcinomas abundantly infiltrated by stromal TILs and invasive front TILs had a higher than median number (≥ 9) of invasive front Tregs. $FOXP3^+$ lymphocytes in peripheral TIL areas were not included in this analysis as there were no statistically significant differences between peripheral TIL number and other variables. Statistical associations are summarized in Table 2.

4. Discussion

Several standardized approaches have been adopted in the histopathological assessment of canine mammary tumors, for example assessing mitotic count in a standard area of 2.37 mm^2 (Meuten et al., 2016) or applying common cut-off values for immunohistochemical markers (Peña et al., 2013). These approaches have improved the reproducibility of data obtained for both research and diagnostic purposes. For this reason, we sought to validate and propose a method, similar to that adopted by the International TILs Working Group for human breast cancer (Salgado et al., 2015), for evaluating TILs in canine mammary tumors. The strength of this method lies in the evaluation of the spatial distribution of TILs as well as the definition of specific cut-off values. In addition to the parameters proposed by the TILs working group (Salgado et al., 2015), we evaluated not only stromal TILs, which are known to have predictive value in women, but also TILs on the invasive front and at the periphery (within the adjacent mammary gland). The latter two were also evaluated in human breast cancer but were not considered to have a predictive value.

Several studies have previously evaluated TILs in canine mammary carcinoma but the use of different cut-off values (Carvalho et al., 2011, Saeki et al., 2012, Kim et al., 2013, Franzoni et al., 2019) and a lack of a specific data related to spatial distribution (Kim

et al., 2013, Franzoni et al., 2019) do not allow comparisons across studies. To evaluate TILs in canine mammary carcinomas, Franzoni et al. (2019) applied a system used in human prostatic adenocarcinomas (Hussein et al., 2009). However, the specific location where TILs were evaluated was not clearly defined in this study. Similarly, Kim and colleagues (2013) used an evaluation system in which the spatial distribution of the TILs was not clearly highlighted (Kim et al., 2013).

Spatial evaluation of TILs is a relevant aspect in breast cancer because stromal TILs are considered the most clinically relevant. Stromal TILs in breast cancer are defined as mononuclear cells (predominantly lymphocytes) present in the tumor stroma between cancer cells without infiltrating tumor cell nests (Kos et al., 2020). Cells that infiltrate tumor nests are defined as intratumoral TILs, characterized by cell-cell contact within tumor nests with no intervening tumor stroma (Kos et al., 2020). Although some studies seem to have assessed TILs inside canine mammary tumors, greater accuracy is needed (Carvalho et al., 2011, Saeki et al., 2012, Kim et al., 2013, Franzoni et al., 2019). In humans, stromal TILs are routinely evaluated and included in diagnostic reports. Although both stromal and intratumoral TILs both correlate with outcome, only stromal TILs are evaluated, as they are more prevalent, more variable in number and more easily assessable than intratumoral TILs (Kos et al., 2020).

TILs are associated with well-known biological predictors of canine mammary carcinomas, such as histological grade and nodal metastasis(Peña et al., 2012, Rasotto et al., 2017). No association was found between these parameters and peripheral TILs in dogs. This is similar to what is known for women with breast cancer (Salgado et al., 2015). However, a possible type I error should be considered for statistical interpretation of metastasis in the lymph nodes because of the small sample size.

The system proposed by the International TILs Working Group includes quantitative scoring. In the present study, TIL evaluation was conducted using a three-tier semiquantitative method that groups the percentage of TILs into three categories (0–10%, 11–40% and 41–90%). This scoring system was prognostically relevant in our study and could be applied in routine diagnostic practice without the aid of image analysis. However, further studies are needed to validate its prognostic role. Although we performed immunohistochemistry to characterized lymphocytes in the TIL population, the semiquantitative assessment of TILs was performed on H-E slides.

Stromal TILs are considered a predictive marker in human breast cancer. In fact, they are associated with a higher frequency of complete pathological response (Denkert et al., 2010), a reduction in the risk of recurrence (Loi et al., 2014) and a prolongation of overall survival (Luen et al., 2018). High numbers of stromal TILs represent a positive predictive factor in HER2-positive and triple-negative breast cancers but not in luminal breast cancers, due to different tumor immunogenicity (Salgado et al., 2015). Conversely, in the canine literature, the degree of lymphocyte infiltration is reported to be higher in mammary carcinoma with lymphatic invasion, high histological grade (Kim et al., 2013) and shorter overall survival (Estrela-Lima et al., 2010, Carvalho et al., 2011, Saeki et al., 2012). However, correlation with molecular classification and response to treatment has not been reported. In our study, a high number of infiltrating TILs was associated with a higher tumor histological grade.

The immune cell populations associated with canine mammary carcinomas may be phenotypically heterogenous and may have both anti-tumor and pro-tumor effects. In dogs, an inflammatory infiltratecharacterized by large numbers of $CD3^+$ and $CD4^+$ T lymphocytes and tumor-infiltrating macrophages was associated with a shorter survival time in a selected case series of triple-negative canine mammary carcinoma (Franzoni et

al., 2019). Abundant $CD4^+$ and Treg cells are both markers of poor prognosis in humans. Treg cells have overlapping TCRrepertoires with naïve $CD4^+$T cells, which suggests that Tregs originate from naïve T cells in situ rather than from recruited Tregs. Blocking the recruitment of $CD4^+$ T cells in human breast cancer significantly reduces the number of intratumoral Tregs and tumor progression, suggesting that intratumoral infiltrating Tregs arise via chemotaxis of circulating naïve CD4 + T cells that differentiate into Tregs in situ (Su et al., 2017). In our study, we found that a subset of the TIL population was composed of Treg lymphocytes that increased in parallel with the overall increase in TIL number. The association between invasive front Tregs and both stromal and invasive front TILs indicates a spatial-specific and selective recruitment of Tregs at the invasive front in order to create a possible immunosuppressive barrier at the tumor front.

Tregs are known to play a role in canine mammary tumor malignancy. Treg-associated histological features include high histological grade, presence of neoplastic emboli, lymph node metastasis and microvascular density (Kim et al., 2013, Carvalho et al., 2016). Similarly, we found a significant association between Tregs and both high histological grade and lymph node metastasis in our study. Considering the role of TILs in adjuvant therapy of triple-negative and HER2 over-expressing tumors, a future direction could be to use a standardized method to evaluate TILs in relation to molecular subtypes and response to medical therapy.

The standardized method of the International TILs Working Group is used worldwide in the diagnosis of human breast cancer (Salgado et al., 2015). TILs have a negative prognostic value in canine mammary tumors, as demonstrated by several studies (Estrela-Lima et al., 2010, Carvalho et al., 2011, Saeki et al., 2012, Kim et al., 2013). Therefore, this standardized method could be included in the diagnostic workflow for canine mammary tumors, in addition to other well-known prognostic parameters such as histological grade

and nodal status. This would provide oncologists with an additional parameter that may be useful in the clinical case management.

5. Conclusions

This study demonstrated that infiltration by stromal TILs and invasive front TILs was associated with increased tumor malignancy. A significant fraction of Treg was present within TILs, and these Tregs may induce immunosuppression, thus promoting tumor progression. The International TILs evaluation system adapted to the canine species is a feasible method of TILs evaluation. In addition, the scoring of TILs may be a further prognostic marker that could be included in diagnostic reports for canine mammary carcinoma. Finally, these data provide useful information in relation to the immune microenvironment associated with canine mammary carcinomas and may help guide therapeutic strategies targeting the immune system.

Conflict of interest statement

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