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Immunohistochemical study of estrogen and progesterone receptors and cell proliferative indexes in canine inflammatory mammary carcinoma: 9 cases

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Submitted October 29th 2007, accepted January 30th 2008,

Abstract

Inflammatory mammary carcinoma (IMC) is a unique form of mammary cancer that affects women and female dogs. Hallmarks of IMC include clinical signs of inflamed affected glands and invasion of dermal lymphatics by tumor cells. Due to locally aggressive behavior and high metastatic rate, prognosis is considered poor. No satisfactory treatment has been suggested in veterinary medicine. The goal of this study was to evaluate the immunoreactivity for estrogen and progesterone receptors and cell proliferative indexes in nine cases of canine IMC. Estrogen and progesterone receptors were negative in all cases. For cell proliferative indexes, the mean number of neoplastic cells staining positive for Ki-67 and PCNA were 4.47% and 20.81%, respectively. Lack of estrogen and progesterone receptor expression, suggesting an undifferentiated phenotype, in conjunction with a relatively high proliferative capacity reflected by Ki-67 and PCNA staining are features typical of many clinically aggressive neoplasms, including canine IMC.

Keywords: Dog, inflammatory mammary carcinoma, estrogen receptors, progesterone receptors, proliferative indices.

Introduction

Canine mammary tumors are common neoplasms and have been reported to account for up to half of all tumors in female dogs (19). Canine mammary tumors share many similarities with breast cancer in human beings, including the high prevalence of adenocarcinomas, frequency of metastasis and progressive disease (19). Inflammatory mammary carcinoma (IMC) is a fulminant form of mammary cancer associated with poor survival rates (4, 5). In women, IMC may present with multiple clinical features including erythema, cellulitis, warmth, ridges, and the absence or presence of a palpable mass (Haagensen's clinical criteria) (5). Although these physical

features are clinically distinct from other mammary tumor subtypes, they do not allow for the definitive diagnosis of IMC (7). In order to confirm the diagnosis of IMC, dermal lymphatic invasion by tumor cells must be identified by histological examination (17).

Not all cases of confirmed IMC require a correlation between clinical appearance and histologic findings (17). IMC may also take the form of breast carcinoma with cellulitis, as well as breast carcinoma without cellulitis, but with histologically confirmed dermal lymphatic invasion (17). Ellis & Teitelbaum (1974) (7) suggest that the term IMC is confusing since clinically it can have inflammation without tumor emboli in dermal lymphatics. Therefore, adenocarcinoma of the breast in a

warm, erythematous, edematous, tender breast does not necessarily mean inflammatory carcinoma (7).

In dogs, the clinical signs of IMC are similar to those of human patients, with the majority of affected canines manifesting inflammation of the affected mammary gland as a hallmark lesion (23). A recent histopathological study of canine IMC demonstrated that the majority of dogs (14/19) had histologically-confirmed dermal lymphatic involvement, similar to the percentage of women with IMC1. Despite many similar clinical findings shared between female dogs and women affected with IMC, blood coagulation disorders appear to be restricted to canines (21, 23).

The assay of ER has become a routine procedure in the clinical evaluation of human breast cancer (2). In humans, estrogen plays an important role in the normal physiology of the mammary gland and has a proliferative effect upon human breast tissue and can contribute to the development of tumor (19). Similarly, hormonal status of female dogs has been demonstrated clinically to influence the development and course of mammary tumors in dogs (19). The actual role of steroid receptors such as estrogen and progesterone in the development of canine mammary tumors remains poorly described in veterinary medicine, although there are similarities between some hormonal aspects of canine and human breast carcinogenesis (9).

Toxicological and epidemiological studies have demonstrated that steroid hormones and their synthetic by-products promote the formation of mammary gland tumor in dogs (16). Estrogen receptors (ER) and progesterone receptors (PR) are present in more than 50% of mammary tumors in dogs (16). Normal mammary glands, mammary dysplasia and benign tumors have higher ER and PR levels than malignant tumors and metastases frequently are negative for both receptors (6, 14). Loss of ER/PR from mammary carcinoma has been associated with decreased cellular differentiation and progression of the disease in human beings and dogs. Thus, ER and PR status also appears to be a useful prognostic indicator for mammary neoplasia in dogs (19).

Undoubtedly, a tumor's growth rate plays an important role in predicting its aggressiveness. A high proliferative rate has been established as an important independent indicator of early relapse in breast carcinoma and is useful in predicting the likely benefit of chemotherapy, whereby tumors with large growth fraction show a significantly better response to treatment than those with a low growth rate (15). The direct clinical evaluation of a tumor's growth rate is not feasible, therefore several measurable kinetic parameters have been identified as prognostic indicators (11). One such prognostic indicator is the measurement of a tumor's growth fraction measured by monoclonal antibody Ki-67 (11). A significant inverse correlation has been demonstrated between the immunohistologically determined ER and Ki-67 count in 74 malignant breast neoplasms in women (15).

A great number of studies on human breast cancer have identified a direct correlation between Ki-67 staining and other prognostic variables such as mitotic index, tumor size, histological grading and lymph node involvement (24). Ki-67 staining correlates well with other proliferative indices, but has been suggested to be more accurate than mitotic index or PCNA staining because Ki-67 is expressed throughout the cell cycle, whereas mitosis represents a very small portion of the whole growth cycle and the half-life of PCNA exceeds the cell cycle time (22).

The relationship between Ki-67 staining and ER and PR content has been suggested to be an inverse correlation (24). The purpose of this study was to evaluate the proliferative indexes using Ki-67 and PCNA, and the ER/PR receptor expression in nine cases of canine IMC.

Materials and Method

Nine cases of canine IMC, confirmed by clinical signs (mammary gland inflammation) and histopathology (dermal lymphatic invasion by neoplastic cells) were selected. Specimens were fixed in buffered 10% formalin, dehydrated, embedded in paraffin, and 3µm thick sections were cut from each mammary neoplasm. The sections were mounted onto slides coated with 3-amino-propyl-triethoxysilane (Sigma, cod. A-3648), deparaffinized in xylene and rehydrated in graded ethanol concentrations.

Antigen retrieval was performed in 10mM citrate buffer in a microwave oven at 750W for 15 minutes. The slides were left to cool to room temperature in the buffer. After 10 minutes washing in running tap water and rinsing in distilled water, endogenous peroxidase was quenched by immersion in a solution of 30% hydrogen peroxide and methanol (50 ml of H₂O₂ and 50 ml of methanol) for 10 minutes. The slides were washed in distilled water then with TRIS (pH 7.4) for 5 minutes, and then incubated with the primary monoclonal antibodies (MABS) in humid chamber for 18 hours at 4°C. The antibody clones and dilutions used were: for progesterone receptor, clone 1A6 (DakoCytomation, California, USA) diluted 1:50; for estrogen receptor, clone 6F11 (Novocastra Laboratories Ltd, Newcastle, UK) diluted 1:50; for Ki-67, clone Ki-S5 (Dako) diluted 1:50; and for PCNA, clone PC10 (Dako) diluted 1:50. The slides were washed in TRIS for 5 minutes and incubated with the LSAB kit (Label Streptoavidin-biotin-peroxidase – Dako, K0690) in humid chamber at room temperature for 30 minutes with each reagent. Visualization was achieved with 3,3'-diaminobenzidine tetrahydrochloride (DAB – Sigma, 5637) in Tris-HCl buffer, then slides were rinsed and counterstained with hematoxylin, dehydrated in graded alcohol concentrations and xylene and mounted with Permount™.

For estrogen and progesterone receptors, a canine mammary ductal adenocarcinoma was used as positive control. The purpose of this positive control case was to verify that the antibodies do react with canine mammary

tissue, so the negative slides were really not reactive with hormone receptors. Ki-67 and PCNA signals were localized in the nuclei and the labeling indexes were scored by counting 1000 cells in 10 to 15 fields, at 400X magnification for each histological section.

Correlation between Ki67 and PCNA labeling indexes were done using unpaired t test (Graph Pad InStat).

Results

In this series, all dogs were intact females 8-14 years of age (median 10.2 years). No breed predilection was observed. Histopathology confirmed poorly differentiated adenocarcinoma with dermal lymphatic invasion by tumor cells in all patients. Clinical signs included erythema (91.7%), mammary firmness (66.7%), bilateral involvement (58.5%), pain (58.5%), mammary enlargement (33.5%), nipple retraction (25%), hind limb edema (25%) and metastasis to lymph nodes (8.5%). All animals were thrombocytopenic, with platelet counts ranging from 30,000 to 95,000/dl (median 48,400/dl). At initial presentation, thoracic radiographs were negative for metastasis in all animals.

Estrogen and progesterone nuclear receptors were negative in all cases of IMC, but not in the control case (a canine ductal mammary adenocarcinoma). The PCNA and Ki-67 labeling indices for each case are shown in Table 1. For PCNA a mean of 20.81±8.63% of the cells were positive (range 8.63%-37.63%) (Fig. 1 - A) and for Ki-67, 4.47±1.97% (range 0.49%-7.7%) (Fig. 1 -B). PCNA and Ki-67 (clone S-5) labeling indexes were statistically different (p=0,0025).

Case	PCNA index	Ki-67 index
No. 1	14.99%	7.7%
No. 2	8.63%	6.50%
No. 3	15.43%	0.49%
No. 4	22.65%	4.10%
No. 5	20.73%	4.27%
No. 6	16.01%	4.81%
No. 7	21.76%	3.92%
No. 8	29.48%	4.08%
No. 9	37.63%	4.42%

Table 1 - PCNA and Ki-67 proliferative labeling indices for each case of IMC.

Discussion

Both clinical signs and histopathological findings are necessary for the accurate diagnosis of IMC (1). The pathologic diagnosis of IMC requires the histological finding of dermal lymphatic tumor invasion, which was demonstrated in all nine cases described in this retrospective study. The purpose of this study was to

evaluate the proliferative indexes by using Ki-67 and PCNA, and the ER/PR receptor expression in nine cases of canine IMC.

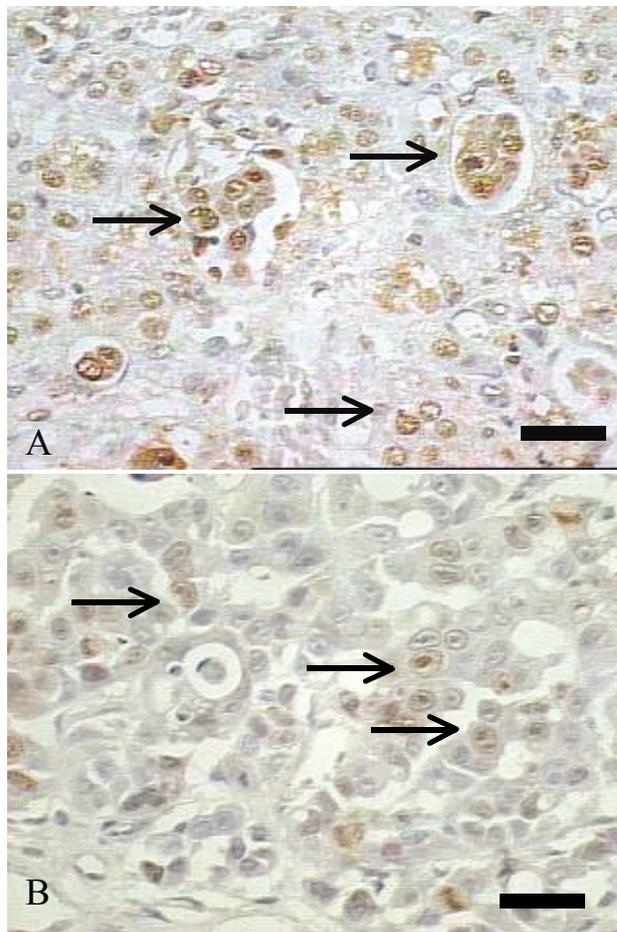


Figure 1: A - Mammary gland, case No. 4. Immunostaining for PCNA in a case of IMC. The arrows show examples of positively stained nuclei. Immunohistochemistry (IHC). Counterstained with Mayer's Hematoxylin. Bar = 20µm. B - Mammary gland, case No. 7. Immunostaining for Ki-67 in a case of IMC. The arrows show examples of positively stained nuclei. Immunohistochemistry (IHC). Counterstained with Mayer's Hematoxylin. Bar = 20µm.

In this study, percentage PCNA positive tumor cells (mean 20.81% for all nine cases) was higher than Ki-67 positive cells (mean 4.47% for all nine cases). This is an expected finding because PCNA has a 20-hour half-life and is detectable throughout the cell cycle (10).

The low values for Ki-67 positive reactions (4.47%) compared to human breast carcinoma snap frozen samples (2.25-63.95%, mean 20.65%) could have occurred because it is conceivable that there is a loss of the antigen recognized by Ki-67 after excision of the tumor and fixation in formalin (3).

In a previous study, AgNOR, Ki-67 (MIB-1 antibody) and PCNA were used to evaluate proliferation 96 canine mammary tumors divided by the histopathological diagnosis (12). The highest indices were found in solid carcinoma (38.6±12.7, for MIB-1 and

42.2±17, for PCNA), complex carcinoma (28.9±23, for Ki-67, MIB-1 and 33±15.2 for PCNA) and invasive/anaplastic carcinoma (23.3±7.8 for Ki-67, MIB-1 and 49.1±13.8 for PCNA) (12). These results showed that the more anaplastic the tumor, the highest PCNA indexes they had, and lower Ki-67 (MIB-1) indexes. In a study of proliferative activity in canine malignant mammary tumors divided by histological stages the Ki-67, MIB 1 index for each group was, 3.8±1.6 (stage 0 – no stromal invasion); 6.5±2.6 (stage 1 – stromal invasion by neoplastic cells) and 11.2±7.5 (stage II – lymphatics embolis) showing that the worst histological behavior had the highest proliferative index (22).

In this series the proliferative indices determined by Ki-67 – clone S5 immunostaining was significantly lower than results reported using Ki-67, clone MIB-1 as in the work done by Lohr *et al.* (1997) (12).

Barnard *et al.* (1987) (3), found no correlation between tumor size and Ki-67 score, in sixty cases of primary breast carcinoma in women, since the relationship between tumor cell proliferation and tumor size is probably influenced by the degree of necrosis, apoptosis and the amount of stromal proliferation present (3).

Raymond and Leong (1989) (15) observed strong positive correlation between Ki-67 count and histological grade of the infiltrating ductal carcinoma in women. The histological grade was inversely correlated with ER count. The Ki-67 count for malignant breast cancer (mean 20.3%) was higher than the benign breast epithelium (mean 2.25%) (15).

There is a positive correlation between the increase recurrence of breast cancer with lower levels of estrogen receptors and an inverse relationship exist between steroid receptor status and S-phase values established from flow cytometry (8). The high proliferative indices are related to the absence of hormonal receptor expression, being in agreement with Gaffney *et al.* (1995) (8), that there is an inverse correlation between steroid receptor status and proliferative indexes.

The findings in canine mammary tumors (14) is in agreement with those of other studies of human breast cancer, indicating that well differentiated tumors can maintain some hormonal regulatory mechanisms and have low proliferation rate (20). Also, human patients with breast cancer containing estrogen or progesterone receptors have a higher survival rate and are more likely to respond to treatment with hormones (19).

In canine mammary tumors, MIB-1 labeling index was higher in malignant compared to benign tumor and MIB-1 labeling index correlated inversely with expression of estrogen receptor-alpha (14). PCNA labeling index was higher, and statistically different from Ki-67 labeling index because PCNA protein has a longer half-life than Ki-67, therefore immunostains more cells than Ki-67. Some PCNA positive cells are no longer in mitosis that is why Ki-67 is a better antibody for proliferation index.

The study of proliferative indexes and hormonal receptor status for IMC provides results consistent with other malignant mammary tumor subtypes. Hormone receptor expression and proliferation indexes have not been previously reported for IMC in either humans and dogs and the findings are consistent with the aggressive behavior of IMC, with lack of hormonal receptors, showing an undifferentiated tumor and therefore more aggressive.

Acknowledgments

We recognize the help of Dr. Timothy Fan and thank him for the great suggestions and editing this paper.

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