RESEARCH ARTICLE Agricultural Scinece Digest, Volume 39 Issue 4: 335-340 (October-December 2019)

A Study on Immunohistochemical Expression of Progesterone Receptor and Cell Proliferation by AgNOR in Canine Mammary Tumors (CMT)

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10.18805/ag.D-4968

ABSTRACT

Mammary gland tumors are the most commonly diagnosed tumors in domestic dogs. Although immunohistochemical methods provide valuable information such as the location and semi-quantitative data of the interested antigens in particular tumors, conventional methods like histopathological diagnosis remain useful and necessary for identification and classification of tumors. In the present study, we combined histopathology with immunohistochemical staining for progesterone receptor (PR) and special staining argyrophilic nucleolar organizer regions (AgNOR) for cell proliferation in canine mammary gland tumors. Twenty-nine dogs with primary mammary gland neoplasms (11 benign and 18 malignant) were included in this study. All 29 tumors were stained with AgNOR but only 15 cases expressed positive PR signals in the nuclei of neoplastic alveolar and ductal epithelial cells. A weak positive correlation (r = 0.2) was observed between PR and AgNOR index of benign tumors and a very weak negative correlation (r = -0.1833) between PR and AgNOR index of malignant tumors suggesting that higher cellular proliferation is associated with low expression of progesterone receptors.

Key words: Mammary tumors, AgNOR staining, Progesterone receptor, Steroid hormones.

INTRODUCTION

Canine mammary neoplasia is a spontaneously occurring disease believed to be influenced by steroid hormones like estrogen and progesterone. Progesterone is essential for the development and growth of the mammary glands, but it also increases the risk of development of mammary neoplasia. Progesterone receptor status (PR) is a marker for hormone responsiveness and disease prognosis in breast cancer. Progesterone receptor negative neoplasms generally have a poor prognosis than progesterone receptor positive neoplasms. The detection of hormone receptors in formalin-fixed, paraffin-embedded (FFPE) samples of canine mammary tumors (CMT) by immunohistochemistry (IHC) started at the end of the 1990s and permitted the identification of positive cells by microscopic observation of labeled nuclei (Geraldes *et al.,* 2000; Graham *et al.,* 1999; Nieto *et al.,* 2000). Studies evaluating the usefulness of estrogen receptor (ER) and progesterone receptor (PR) as prognostic factors in dogs with mammary tumors are uncommon and include two retrospective (Chang *et al.,* 2009; Millanta *et al.,* 2005) and two prospective reports (Martin de las Mulas *et al.,* 2005; Nieto *et al.,* 2000). Moreover, their results are inconclusive: malignant CMT with ER expression had a better prognosis in 1 prospective multivariate study (Nieto *et al*., 2000) but not in another (Martin de las Mulas, 2005), while PR expression was associated with increased overall survival in a retrospective univariate study (Chang *et al.,* 2009). Ninety-five percent of normal canine mammary tissues contain progesterone and/or estrogen receptors and over 50% of canine mammary tumors express estrogen and progesterone receptors (Thuroczy *et al.,* 2007).

The hormonal dependency of canine mammary tumors generally decreases with increasing malignancy and higher cellular proliferation occurs in malignant tumors with low expression of progesterone receptors (Geraldes *et al.,* 2000). Although there are many methods for detecting cell proliferation, only a few methods are applicable on formalin-fixed paraffin-embedded tissues. Among these, the estimation of the growth fraction by means of the immunohistologic demonstration of the proliferation associated nuclear antigens Ki-67 and PCNA as well as the AgNOR method are relatively easy to perform and are therefore being increasingly used for diagnostic and prognostic purposes (Lohr *et al.,* 1997).

Argyrophilic nucleolar organizer regions (AgNORs) are silver-stained proteins co-localized with DNA loops of the nucleolus. Because AgNORs are related to DNA synthesis and metabolic activities, their size and number are used to reflect nuclear activity. High levels of AgNOR proteins indicate a high rate of cell proliferation and poor prognosis of neoplasms (Madewell, 2001).

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How to cite this article: P.L. Leena Rajathy, K.C. Varshney, M.G. Nair, A.W. Lakkawar and B.R. Kumar (2019). A Study on Immunohistochemical Expression of Progesterone Receptor and Cell Proliferation by AgNOR in Canine Mammary Tumors (CMT). Agricultural Science Digest. 39(4): 335-340.

Source of Support: Nil

Conflict of support: None

Submitted: 17-06-2019 **Accepted:** 26-08-2019 **Published:** 06-12-2019

As approximately 50% of mammary gland tumors in dogs appear to be malignant (Bostock, 1986; Bronden *et al.,* 2010), the detection of novel canine tumor markers with a value for prognosis or targeted therapy is the focus of research (Mohr *et al.,* 2016). There are some recognized, well-accepted prognostic factors of malignant mammary neoplasms in the dog such as tumor size, lymph node status, histologic type, histologic malignancy grade, degree of nuclear differentiation and distant metastasis. There are also other proven or controversial host and tumor prognostic factors such as HER-2, p53, PCNA and Ki-67, and the number of new ones is steadily increasing (Martin de las Mulas *et al.,* 2005). Until now, the most studied and reliable biomarkers of CMT are antigen Ki-67 (Ki-67), endothelial growth factor receptor, human epidermal growth factor receptor 2 (HER-2), estrogen receptor, progesterone receptor and cyclooxygenase 1 (COX-2), which can be detected in both serum and tissue samples using different molecular methods (Kaszak *et al.,* 2018).

The aim of the present study was to determine whether there is a correlation between the expression of progesterone receptor and the rate of cellular proliferation detected by AgNOR in neoplastic canine mammary tissues.

MATERIALS AND METHODS

Animals and Samples

Samples of mammary tumors were collected from 29 dogs that were presented to the Department of Surgery and Radiology (Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Pondicherry, India) for clinical evaluation and treatment of mammary neoplasia. Out of the 29 dogs with mammary tumors, 28 were females and one was a male German Shepherd dog (GSD) of 10 years of age. The dogs ranged in age from 5 to 15 years (10.21± 2.91) and were of various breeds including mongrels (Table 1).

Histological Examination

The tissue samples fixed in 10% neutral buffered formalin were processed for histopathological examination by routine paraffin embedding technique and microtomy (Luna, 1968). Five-micron thick sections were stained by H&E for detailed histopathological study and classification of tumors. Canine mammary tumors were classified using the WHO histological classification (Hampe and Misdorp, 1974).

Table 1: Breed distribution of dogs with mammary tumors (n=29).

Breed	No. of dogs
German Shepherd	8
Spitz	10
Tibetan Terrier	2
Pomeranian	2
Weimaraner	1
Mongrel	6

Table 2: Age-wise incidence of mammary tumors in dogs.

Age in years	No. of cases	Percent
0–3	0	0
4–7	6	21
8–11	12	41
12–15	11	38

Histochemistry- AgNOR Staining

Formalin fixed paraffin embedded tissue sections of the 29 tumors were stained according to the protocol of the standardized AgNOR method (Jelesijevic *et al.,* 2003) with minor modifications as detailed below:

1) The sections were deparaffinized in two changes of xylene for 10 min each, hydrated in graded alcohol and washed in ultrapure water for 5 minutes.
2) Pre-treatment of the sections was carried out by pressure cooking for 20 minutes in 10 mM citrate buffer, pH 6.0.
3) Sections were cooled to room temperature and washed for 2 minutes in ultrapure water.
4) Fresh solutions of 33% silver nitrate and 2% gelatin in 1% formic acid were prepared in ultrapure water.
5) The pre-treated sections were stained with two parts of freshly prepared 33% silver nitrate solution mixed with one part of 2% gelatin in 1% formic acid for 30–45 minutes (depending on the staining intensity of each tissue block) at room temperature in the dark in a humidified chamber.
6) The stained sections were rinsed thoroughly with ultrapure water and fixed in 2% sodium thiosulfate solution for 5 minutes.
7) Finally the sections were washed in distilled water, dehydrated in graded ethanol, cleared in xylene and mounted with DPX.

Morphometric Analysis of AgNORs

Morphometric analysis and quantification of AgNORs were performed with the Image-Pro PLUS 6.0 software (Media Cybernetics, USA) on all the 29 tumors. Calibration and threshold determination for the AgNORs were done once for each section using a 40x objective. On each slide, 250 tumor cells were measured within the area most typical for the histopathologic diagnosis. The mean nuclear area (NA) and mean AgNOR area/nucleus (AA) for each sample were calculated as described by (Munakata and Hendricks, 1994):

$$\text{Nuclear area } (\mu m^2) = \frac{\sum (\text{nuclear area})}{\text{Number of nuclei}}$$

$$\text{NOR area } (\mu m^2) = \frac{\sum (\text{AgNOR area})}{\text{Number of nuclei}}$$

The mean NOR number (NN) was calculated. The NOR percentage nuclear area (NPNA) or NOR index was calculated for each specimen as follows:

$$\text{NPNA } (\%) = \frac{\text{AgNOR area}}{\text{Nuclear area}} \times 100$$

Immunohistochemistry

After H&E staining and classification of the mammary tumor, adjacent paraffin sections (4 µm thick) were mounted on Histogrip® coated slides and dried for 24 hours at 37°C. Immunostaining was performed using Ready-to-use Mouse anti-PR (Human, Clone PR88) (Biogenex, USA, Cat. No AM 328-5M (RTU-M)) and Super Sensitive Polymer-HRP Detection System (BioGenex, USA Kit Code: QD400-60K).

Immunostaining Procedure

1) Sections were deparaffinized and hydrated.
2) Tissue sections were first subjected to heat antigen retrieval by microwaving in citrate buffer 0.01 M, pH 6.0 for 10 minutes at 800 W plus two periods of 10 minutes at 320W and cooled in buffer for 20 minutes.
3) Endogenous peroxidase activity was inhibited with peroxide block for 10 minutes at RT.
4) Sections were washed with TBS and gently blotted.
5) Power Block™ was applied to cover the section and incubated for 10 minutes at RT. Sections were then gently blotted.
6) Primary antibody was added to cover the sections and the slides were incubated for 1 hour at RT. Sections were rinsed well with TBS and gently blotted.
7) Super Enhancer Reagent was added and the sections were incubated for 20 minutes at RT and then rinsed well with buffer.
8) Poly-HRP Reagent was added and the sections were incubated for 30 minutes at RT and then rinsed well with buffer.
9) DAB substrate solution was added and the sections were incubated for 5 minutes at RT until acceptable color intensity (light brown) reached and then rinsed well with deionized water.
10) The slides were counterstained with Mayer's hematoxylin for 5-10 minutes, and rinsed with tap water.
11) Sections were dehydrated in ascending grades of ethanol, cleared in xylene and mounted using DPX.

Image Analysis

Image analysis was carried out on all immuno histochemically stained sections. All images were captured using a binocular Olympus BX41 microscope (Olympus Co., Japan) fitted with an Evolution LC digital camera. The digital images were analyzed using a semi-automated image processing and analysis software, Image-Pro® PLUS 6.0 (Media Cybernetics, USA). The software performs automatic measurement of areas defined using an interactive threshold editing functions.

Quantification of Immunostaining

Immunohistochemistry enables the detection of an antigen. This antigen is visualized by the brown precipitate of 3-3'-diaminobenzidine (DAB). Using the Image-Pro PLUS 6.0 (Media Cybernetics, USA), the area representing this brown coloration can be measured. Five random objective fields of PR stained cells within the mammary neoplasms were captured on digitalized images at a final magnification of 400x. The percentage of positive cells was calculated from minimum of 500 cells counted.

Statistical Analysis

Correlation between variables was calculated using Spearman's Rank Correlation test (Fowler and Cohen, 1990). The level of critical significance was considered to be $p<0.05$.

RESULTS AND DISCUSSION

Eleven out of the 29 mammary tumors were classified as benign (1 papillary cystadenoma, 1 fibrocystadenoma, 1 duct papilloma, 2 fibroadenoma and 6 mixed mammary tumors) and 18 as malignant tumors (2 papillary adenocarcinoma, 5 papillary cystadenocarcinoma, 3 solid adenocarcinoma, 3 complex adenocarcinoma, 2 were carcinosarcoma, 1 squamous cell carcinoma, 1 sebaceous adenocarcinoma and 1 hemangiopericytoma) by histopathology.

Dorn *et al*. (1968) found pure-bred dogs to be over-represented among mammary tumor cases and postulated a genetic influence in its etiology. In the present study also, the occurrence of mammary tumors in pure breeds was higher compared to mongrels and cross breeds. Among the pure breeds, Spitz had the highest occurrence of mammary neoplasms followed by GSD. Priya (2005), Anil (2007), Jain and Raghunath (2007) also reported the highest occurrence of mammary neoplasms in Spitz breed. In the present study, the highest occurrence in Spitz could be attributed to the higher number of Spitz population in the Pondicherry region and the preference of pet owners to rear Spitz breed compared to other dog breeds. However, this aspect needs further detailed investigation.

In the present study, one of the parameters assessed was the age-wise incidence of mammary tumors. Several workers report the maximum incidence of neoplasm

Table 3: AgNOR parameters of mammary tumors in dogs (n=29).

Type of neoplasm (no.)	AgNOR count	AgNOR area (µm²)	Nuclear area (µm²)	NPNA/AgNOR index
BENIGN				
Adenoma (2)	1.616±0.017	1.547±0.722	15.196±2.977	9.905±2.814
Duct papilloma (1)	1.184±0.511	0.477±0.542	7.533±2.372	6.325±1.112
Fibroadenoma (2)	1.776±0.781	1.160±0.793	13.349±6.433	8.213±1.980
Benign mixed tumor (6)	1.583±0.218	1.072±0.315	16.463±1.381	6.461±1.492
MALIGNANT				
Papillary adenocarcinoma (2)	1.72±0.238	0.926±0.069	15.294±2.590	6.104±0.580
Papillary cyst- adenocarcinoma (5)	1.917±0.331	1.028±0.323	18.331±2.769	5.516±1.161
Solid adenocarcinoma (3)	1.734±0.201	1.342±0.582	17.772±2.468	7.981±4.73
Complex adenocarcinoma (3)	1.497±0.046	1.107±0.327	15.922±0.818	6.974±2.187
Carcinosarcoma (2)	2.631±1.254	2.342±0.608	21.857±10.029	11.262±2.386
Sebaceous adenocarcinoma (1)	1.75±0.21	1.256±0.88	16.685±4.79	7.53±0.69
Squamous cell carcinoma (1)	1.29±0.56	0.755±0.08	12.189±0.75	6.24±1.00
Hemangiopericytoma (1)	1.94±1.13	2.177±0.25	14.973±0.60	14.58±1.97

Table 4: Immunostaining of PR in benign and malignant mammary tumors of dogs (n=29).

	Tumor type	n	No. positive	Mean % positivity
1.	BENIGN			
	Adenoma	2	1	44.4%
2.	Duct papilloma	1	1	18.5%
3.	Fibroadenoma	2	1	51.4%
4.	Benign mixed tumor	6	3	55.2%
1.	MALIGNANT			
	Papillary adenocarcinoma	2	1	61.9%
2.	Papillary cystadenocarcinoma	5	3	59.8%
3.	Solid adenocarcinoma	3	1	17%
4.	Complex adenocarcinoma	3	1	32.3%
5.	Carcinosarcoma	2	0	_
6.	Sebaceous adenocarcinoma	1	1	19.4%
7.	Squamous cell carcinoma	1	1	43.5%
8.	Hemangiopericytoma	1	1	35.8%
Total		29	15	

during the 6 to 10 year range (Mitchell *et al.,* 1974; Moulton, 1990). In the present study, the highest incidence of mammary neoplasm was recorded in the age group of 8–11 years (41%) (Table 2). Jain and Ragunath (2007) also recorded highest incidence (30%) of mammary tumors in the age group of 8–10 years. No tumor was recorded in the age group of 0–3 years, indicating that mammary tumor is a disease of older dogs. Mammary tumors in the dog are hormone dependent, and the risk of appearance increases after each estrous cycle. This influence of steroid hormones could not be established in the present study because of the lack of spaying details or cycling stage of the dogs involved.

The utility of AgNOR as a proliferation marker in canine mammary neoplasms has been reported by several workers (Juntes and Pogacnik, 2000; Jelesijevic *et al.,* 2003 and Reddy *et al.,* 2007). After silver staining, the AgNOR could be seen as discrete dots, single to multiple in center or towards periphery of the nuclei (Figs 1 and 2). Morphometric studies made in the AgNOR stained sections of the 29 canine mammary neoplasms are detailed in Table 3.

In the present study, AgNOR counts of malignant tumors were significantly higher than that of benign tumors, as reported by Reddy *et al.* (2007) and Kumar *et al.* (2010), which prove that increased number of AgNOR correlates with increased cellular proliferation. It was also observed in the present study that among the benign tumors, a higher count was recorded in fibroadenoma and among malignant tumors in carcinosarcoma.

In the present study, all tumors showed AgNOR staining but only 15 (52%) cases expressed positive PR signals in the nuclei of neoplastic alveolar and ductal epithelial cells (Figs 4 and 5). Also, PR was expressed in the nuclei of epithelial cells in both benign and malignant tumors, whereas the cartilage and bone cells were negative which concurred with the report by Geraldes *et al.* (2000). Additionally, the nuclei of a few spindle shaped stromal cells also expressed PR as have been reported by Martin de las Mulas *et al.* (2005). PR positivity ranged from 17–72%. Expression of PR immunostaining in case of benign and malignant tumors is represented in the Table 4.

About 55% (6/11) of benign tumors and 50% (9/18) of malignant tumors expressed PR. Among the benign tumors, benign mixed tumor expressed high percentage of PR immunostaining and among the malignant tumors papillary adenocarcinoma expressed the maximum immunostaining. In this study, there was no significant difference ($p>0.05$) in PR expression between benign and malignant tumors which was in accordance with the findings by Toniti *et al*. (2009).

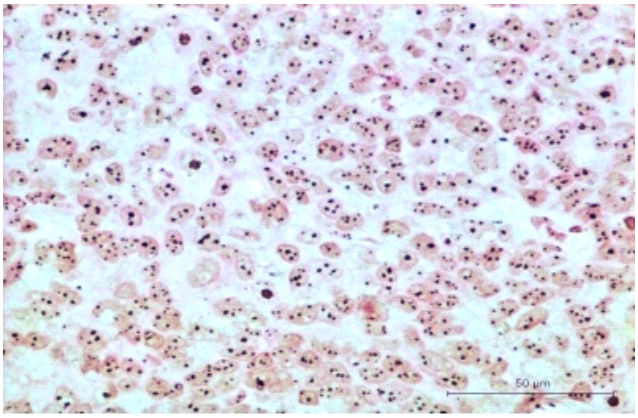


Fig 1: Solid adenocarcinoma in dog mammary gland showing nucleolar organizer regions as discrete black dots of variable size inside the nuclei. Silver stain×400.

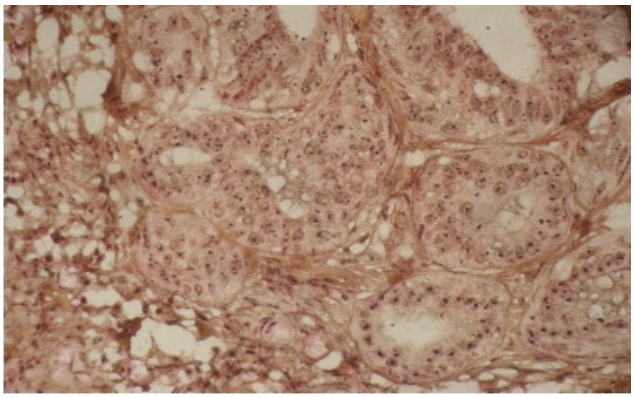


Fig 2: Mixed mammary tumor in dog mammary gland showing nucleolar organizer regions as discrete dots inside the nuclei of proliferating epithelial cells. Silver stain×400.

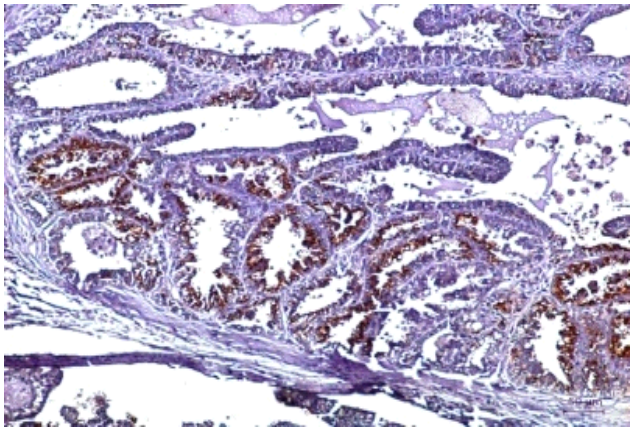


Fig 3: Papillary cystadenocarcinoma in dog mammary gland showing strong progesterone receptor (PR) expression in the nuclei of neoplastic epithelial cells. Immunoperoxidase/DAB substrate/ Mayer's hematoxylin counterstain ×100.

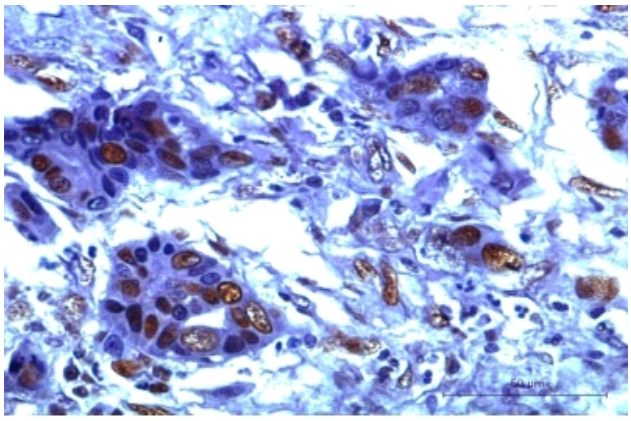


Fig 4: Fibroadenoma in dog mammary gland showing progesterone receptor (PR) expression in the nuclei of epithelial and stromal cells. Immunoperoxidase/DAB substrate/Mayer's hematoxylin counterstain ×400.

In the present study, a positive correlation (r =0.2) has been observed between PR and AgNOR index of benign tumors and a very weak negative correlation (r = -0.1833) between PR and AgNOR index of malignant tumors suggesting that as there is higher cellular proliferation as occurring in malignant tumors, there is low expression of progesterone receptors. In other words, the hormonal dependency of canine mammary tumors generally decreases with increasing malignancy. The malignant tumors that are negative for progesterone receptors are proliferating at higher rates than the positive tumors. This finding was in agreement with the report by Thuroczy *et al*. (2007) who reported a negative correlation between PR and Ki-67 LI of canine malignant mammary tumors. The present study also revealed that there was no significant difference in PR expression between benign and malignant tumors which was in contradiction to the reports by Thuroczy *et al.* (2007). However, this could be due to the less number of mammary tumor cases analyzed in the present study.

CONCLUSION

Canine mammary tumors are challenging for both clinicians and pathologists because their behavior and prognosis are difficult to predict. However, prognosis and therapy of mammary tumors can be carried out based on the hormonal receptor expression. From the results of the present study, it could be concluded that the malignant tumors that are negative for progesterone receptors proliferate at higher rates than that are positive, suggesting that the progression towards malignancy in spontaneous canine mammary tumors is accompanied by a decrease in hormonal steroid dependency and an increase in autonomous growth.

ACKNOWLEDGMENT

The authors are thankful to the Dean, RIVER, Pondicherry for providing all the facilities for the conduct of the study. The authors thankfully acknowledge the help rendered by the staff of the Department of Veterinary Surgery and Radiology, RIVER, who helped in the collection of tissue samples for this study.

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ISSN: 0976-0547 (Online), 0253-150X (Print), Naas Rating: 4.21