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

P.L. Leena Rajathy, K.C. Varshney, M.G. Nair, A.W. Lakkawar and B. Ramesh Kumar

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 (Martín 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 a prospective multivariate study (Nieto et al., 2000) but not in another (Martín 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).

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.
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 (10.2±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).

**Histochemistry- AgNOR Staining**

Formalin fixed paraffin embedded tissue sections of the 29 tumors were stained according to the protocol of the standardized AgNOR method (Jesesijevic 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):

\[
\text{Nuclear area (µm}^2\text{)} = \frac{\Sigma \text{(nuclear area)}}{\text{Number of nuclei}}
\]

\[
\text{NOR area (µm}^2\text{)} = \frac{\Sigma \text{(AgNOR area)}}{\text{Number of nuclei}}
\]

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 sections 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 neoplasms...
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).

### 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%             |
| Duct papilloma              | 1  | 1            | 18.5%             |
| Fibroadenoma                | 2  | 1            | 51.4%             |
| Benign mixed tumor          | 6  | 3            | 55.2%             |
| 1. MALIGNANT                |    |              |                   |
| Papillary adenocarcinoma    | 2  | 1            | 61.9%             |
| Papillary cystadenocarcinoma | 5  | 3            | 59.8%             |
| Solid adenocarcinoma        | 3  | 1            | 17%               |
| Complex adenocarcinoma      | 3  | 1            | 32.3%             |
| Carcinosarcoma              | 2  | 0            |                   |
| Sebaceous adenocarcinoma    | 1  | 1            | 19.4%             |
| Squamous cell carcinoma     | 1  | 1            | 43.5%             |
| Hemangiopericytoma          | 1  | 1            | 35.8%             |
| Total                       | 29 | 15           |                   |

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.
In this regards you may please reply of the reviewer comments mail.

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 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.

REFERENCES

Anil, A. (2007). Identification of stem cell lineage cd44+/cd24- and apoptotic marker in mammary tumours of dogs. M.V.Sc. Thesis, TANUVAS, Chennai, India.

Bostock, D.E. (1986). Canine and feline mammary neoplasms. The British Veterinary Journal. 112: 506-15.

Bronden, L.B., Nielsen, S.S., Toft, N., Kristensen, A.T. (2010). Data from the Danish veterinary cancer registry on the occurrence and distribution of neoplasms in dogs in Denmark. The Veterinary record. 166(19):586–90.

Chang, C.C., Tsaì, M.H., Liao, J.W. (2009). Evaluation of hormone receptor expression for use in predicting survival of female dogs with malignant mammary gland tumors. Journal of the American Veterinary Medical Association. 235:391–396.

Dorn, C.R., Taylor, D.O.N., Schneider, R., Hibbard, H.H. and Kluber, M.R. (1968). Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II. Cancer morbidity in dogs and cats from Alameda Country. Journal of the National Cancer Institute. 40: 307-318.

Fowler, J. and Cohen, L. (1990). Practical Statistics for Field Biology. John Wiley & Sons, England.

Geraldes, M., Gartner, F. and Schmitt, F. (2000). Immunohistochemical study of hormonal receptors and cell proliferation in normal canine mammary glands and spontaneous mammary tumours. Veterinary Record. 146: 403-406.

Graham, J.C., O’Keefe, D.A. and Gelberg, H.B. (1999). Immunohistochemical assay for detecting estrogen receptors in canine mammary tumors. American journal of veterinary research. 60:627–630.

Hampe, I.F. and Misdorp, W. (1974). Tumors and dysplasias of the mammary gland. Bulletin of the WHO. 50: 111-133.

Jain, V. and Ragunath, M. (2007). Incidence of canine mammary neoplasms in and around Ludhiana. Punjab Veterinary Journal. 5: 42-43.

Jelešijević, T., Jovanović, M., Knežević Milijana and Aleksic-Kovacevic sanja (2003). Quantitative and qualitative Analysis of AgNOR in Benign and Malignant canine Mammary Gland Tumors. Acta Veterinaria. 53: 353-360.

Jentes, P. and Pogacnik, M. (2000). Morphometric analysis of AgNORs in tubular and papillary parts of canine mammary tumors. Veterinary Pathology. 22:185-192.

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

Kaszak, I., Ruszczak, A., Kanafa, S., Kacprzk, K., Król, M. and Jurka, P. (2018). Current biomarkers of canine mammary tumors. Acta Veterinaria Scandinavica. 60:66.

Kumar, P., Kumar, R., Pawajya, R.V.S., Madhu, B.P. (2010). Diagnostic significance of mitotic index and AgNOR count in canine mammary tumours. Brazilian Journal of Veterinary Pathology. 3: 41-45.

Lohr, C.V., Teifke, J.P., Failing, K. and Weiss (1997). Characterization of the proliferation state in canine mammary tumors by the standardized AgNOR method with postfixation and immunohistologic detection of Ki-67 and PCNA. Veterinary Pathology. 34: 212-221.

Luna, L.G. (1968). Manual of histologic staining methods of the Armed Forces Institute of Pathology. Mc Graw Hill, New York, pp 32-46.

Madewell, B.R. (2001). Cellular proliferation in tumors: A review of methods, interpretation, and clinical applications. Journal of Veterinary Internal Medicine. 15: 334-340.

Martin de las Mulas, J., Millan, M.Y. and Dios, R. (2005). A prospective analysis of immunohistochemically determined estrogen receptor α and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. Veterinary Pathology. 42: 200-212.

Milanta, F., Calandrella, M., Bari, G., Niccolini, M., Vannozzi, I. and Poli, A. (2005). Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. Research in Veterinary Science. 79: 225-232.

Mitchell, L., de la Iglesia, F.A., Wenkoff, M.S., van Dreumel, A.A. and Lumb, G. (1974). Mammary tumors in dogs: survey of clinical and pathological characteristics. Canadian Veterinary Journal. 15: 131-138.

Moulton, J.E. (1990). Tumors of the mammary gland. In: Tumors in Domestic Animals. 3rd edn. (Ed.) J.E. Moulton. Berkeley, University of California Press.

Mohr, A., Lüder Ripoli, F., Hammer, S.C., et al. (2016). Hormone Receptor Expression Analyses in Neoplastic and Non-Neoplastic Canine Mammary Tissue by a Bead Based Multiplex Branched DNA Assay: A Gene Expression Study in Fresh Frozen and Formalin-Fixed, Paraffin-Embedded Samples. PLOS One. 11(9):e0163311.

Munakata, S. and Hendricks, J.B. (1994). A multilabeling technique for simultaneous demonstration and quantitation of Ki-67 and nucleolar organizer regions (AgNORs) in paraffin-embedded tissue. Journal of Histochemistry and Cytochemistry. 42: 789-793.

Nieto, A., Pena, L. and Perez-Alenza, M.D. (2000). Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. Veterinary Pathology. 37:239–247.

Priya, S. (2005). Comparison of nipple aspirate fluid cytology with other routine tests in the diagnosis of canine mammary tumours. M.V.Sc thesis. TANUVAS, Chennai, India.

Reddy, G.B.M., Kumar, R., Sharma, A.K. and Ravindran, R. (2007). Canine mammary tumors: study on cell proliferative markers. Proceedings of XXIV Annual Conference of Veterinary pathologists. Oct. 1-3, Trupati, India.

Toniti, W., Buranasinsup, S., Kongcharoen, A., Charoonrut, P., Puchadapirom, P., and Kasorndorkbua, C. (2009). Immunohistochemical Determination of Estrogen and Progesterone Receptors in Canine Mammary Tumors. Asian Pacific Journal of Cancer Prevention. 10: 907-911.

Thuroczy, J., Reisvaag, G.J., Perge, E., Tibold, A., Szilagyi, J. and Balogh, L. (2007). Immunohistochemical detection of Progesterone and Cellular Proliferation in Canine Mammary Tumors. Journal of Comparative Pathology. 137: 122-129.