The Pharma Innovation Journal 2022; SP-11(5): 233-237

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(5): 233-237
© 2022 TPI
www.thepharmajournal.com
Received: 13-03-2022
Accepted: 15-04-2022

Dr. K Rama Krishna
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Dr. M Jeevana Latha
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Dr. M Lakshman
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Dr. M Usha Rani
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Dr. Y Ravi Kumar
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Dr. B. Swathi
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author
Dr. K Rama Krishna
Department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Expression pattern of PCNA and Ki-67 biomarkers in canine mammary tumours

Dr. K Rama Krishna, Dr. M Jeevana Latha, Dr. M Lakshman, Dr. M Usha Rani, Dr. Y Ravi Kumar and Dr. B. Swathi

Abstract
In the present study 21 Canine mammary tumor (CMT) samples were collected from Department of Veterinary Surgery and Radiology, College of Veterinary Science, Rajendranagar and from various Veterinary clinics in and around Hyderabad. The CMT samples were collected in 10 per cent neutral buffered formalin (NBF) for the histopathology and immunohistochemistry and Tumour tissues were processed by routine paraffine embedding method. H & E staining was performed and tumours were classified based on descriptive morphology and histogenesis. Proliferation kinetics were studied with immunohistochemistry using PCNA and Ki-67 antibodies with a view to use the techniques in differentiating benign and malignant tumours. IHC was carried out on paraffin sections mounted on poly L-lysin coated slides and mean number of positive cells were counted and the values were subjected to standard statistical methods and results were drawn. Out of 21 CMTs studied 16 (77.20%) were malignant and 5 (23.80%) were benign. Out of 16 malignant tumours malignant mixed mammary tumours (25%) were seen predominantly followed by papillary adenocarcinoma (18.75%), tubular adenocarcinoma (12.5%), solid carcinoma (12.5%), cavernous hemangionma (12.5%), squamous cell carcinoma (6.25%), papillary cystic adenocarcinoma (6.25%), fibrosarcoma (6.25%). Out of 5 benign tumours lipoma (40%), soft tissue hyperplasia (40%) were predominantly seen followed by fibroadenoma (20%). The mean PCNA positive nuclei in different tumour tissues varied from 3.24±0.06 to 52.35±7.27. The highest index was observed in solid carcinomas and the lowest index was observed in soft tissue hyperplasia. The mean number of PCNA positive nuclei in benign tumours (5.42±1.48) was significantly (P<0.05) lower than the malignant tumours (39.45±3.84). The mean Ki-67 positive nuclei in different tumour tissues varied from 1.04±0.06 to 47.7±5.26. The highest index was observed in Malignant mixed mammary tumor and the lowest index was observed in soft tissue hyperplasia. The mean number of Ki-67 positive nuclei in benign tumours (6.25±1.51) was significantly (P<0.05) lower than the malignant tumours (32.95±3.16). In conclusion high PCNA and Ki-67 counts were associated with malignancy.

Keywords: Canine mammary tumours, PCNA, Ki-67, immunohistochemistry

Introduction
Neoplasms in dogs are twice more frequent in comparison with humans. Due to their short life span, rapid progression of the disease and a large number of potential foci (ten mammary glands) proving them as excellent models for understanding human breast cancer than the traditional rodent models (Hellmen, 1996) [1]. Canine mammary tumors (CMT) are the second most (25-50%) frequently encountered spontaneous mixed type of tumors which could be either benign or malignant neoplasms following those derived from skin (Benjamin *et al.*, 1999) [2]. Based on the histopathological and biological criteria (from follow up studies), Misdorp (1974) [3] estimated approximately 30 per cent of surgically removed mammary tumors are malignant. Canine mammary tumors with apparent signs of malignancy, such as pronounced infiltrative growth involving several mammary glands associated with edema, ulcerated skin and lymph node metastasis, are easy to an experienced clinician to suspect the malignancy. However, because most of the CMTs are not at an advanced stage of development, when first detected by owners or clinicians, the potential biological behavior (hyperplasia, benign proliferation, malignant transformation) of these tumors is difficult to interpret. Hence, these tumors need to have a better diagnostic tools to increase the possibility of adequate treatment (Hellmen *et al.*, 1993) [4].
Tumour markers are biochemical indicators of the presence of tumour, which includes cell surface antigens, cytoplasmic proteins, cell adhesion molecules, enzymes and hormones (Sivaseelan, 2021) [5].

Several authors have proposed the dog as a model for human mammary neoplasia, based on many similarities that have been noted between two species (Gilbertson *et al.,* 1983) [6]. Due to high similarities of the CMTs with human breast cancers (HBC), human biomarkers of HBC can also detect the CMTs. Effective biomarkers for cancer screening facilitate disease identification in sub-clinically affected patients and lead to subsequent improvements in clinical outcome (Henry, 2010) [7].

In human neoplasms, immunohistochemical markers measure the kinetic parameters of the cells and have been used successfully as prognostic indicators (Chandravathi, 2008) [8]. Two of such markers are Proliferating cell nuclear antigen (PCNA) and Ki-67.

PCNA is an auxiliary protein of DNA polymerase delta, which is expressed in the nuclei of cells during the DNA synthesis phase (S-phase) of cell division cycle. It is involved in the DNA repair process, cell cycle control, chromatin assembly and the RNA transcription. Hence, it's level correlates directly with the cell proliferation (Kaszak *et al.,* 2018) [9].

Ki-67 antigen is a nuclear protein highly expressed in proliferation cells prior to mitosis. For humans, immunohistochemical determination of Ki-67 expression in mammary carcinomas has been indicated as a prognostic marker for relapse-free survival time (Abdelmegeed *et al.,* 2018) [20]. It has highest expression in the mitotic phase (M phase) and its expression disappears rapidly after mitosis. In gap phase (G_0 phase) it is undetectable (Kaszak, 2018) [9].

Screening for premalignant lesions or early invasive disease likely to have the potential to reduce mortality from cancer. Because of their ease of measurement, several biomarkers have been evaluated or/and are currently undergoing evaluation as screening tests for early malignancy (Duffy 2015) [10]. With this emerging need this present study was designed to know the expression pattern of PCNA and Ki-67 in canine mammary tumours for the early detection of malignancy.

Materials and Methods

Canine mammary tumors (CMT) and blood samples were collected from Department of Veterinary Surgery and Radiology, College of Veterinary Science, Rajendranagar and also from various Veterinary clinics in and around Hyderabad. The mammary tumor samples were collected in 10 per cent neutral buffered formalin (NBF) for the histopathology and immunohistochemistry.

Mammary tumor samples for histopathology were preserved in 10 per cent NBF. Routine tissue processing had been carried out (Luna, 1968). Sections of 5 micron thickness were taken and stained with haematoxylin and eosine (H & E) stains. (Luna *et al.,* 1968 and Bancroft *et al.,* 1996) [11].

Immunohistochemistry of PCNA was carried out by using the staining procedure prescribed by Sivaseelan (2021) [5]. Chemicals were obtained from Biomarq labs, L.B. Nagar, Hyderabad. Mounted 3 – 4 µm thick paraffin section on slides coated with poly- L lysine and dried at 56 ºC overnight. Sections were deparaffinized in xylene and washed with isopropyl alcohol (IPA). Then washed the sections under running tap water for 10 min. Then placed the slides in microwaveable plastic couplin jars filled with 250 mL of 0.01 M citrate buffer (pH - 6) for antigen retrieval. Then heated the jar in pressure cooker for 2 min and placed in coupling jar for 30 min at room temperature. Then washed the sections with PBS for 15 min. Then overlayed the slides with peroxide block solution for 10 min. Washed the sections with PBS (5min x 2). Then incubated the slides with primary antibody (PC-10) for 1 h. Washed the sections with PBS (5 min). Incubated the slides with secondary antibody (Anti-antibody for 30 min). Washed the sections with PBS (5 min). Treated the sections with 3,3[1] diaminobenzidine tetrahydrochloride (DAB) and buffer substrate for 5-8 min. Then washed with PBS for 5 min. Then washed with tap water. Then counter stained with haematoxylin. Then washed with water and then alcohol and cleared in xylene. Then examined under light microscope (LM).

Positive cells were identified by the presence of brickred colour in the nucleus and cytoplasm (Non-mitotic cells- expression of PCNA was restricted to nucleus and in mitotic cells expression was with both nucleus and cytoplasm) and the PCNA Index was calculated with the below formula.

$$\text{PCNA Index} = \frac{\text{No. of positive cells/labeled nuclei}}{\text{Total number of cells}} \times 100$$

Immunohistochemistry of Ki-67 was carried out by using the staining procedure prescribed by Sivaseelan (2021). Chemicals were obtained from Biomarq labs, L.B. Nagar, Hyderabad. Mounted 3 – 4 µm thick paraffin section on slides coated with poly- L lysine and dried at 56 ºC overnight. Sections were deparaffinized in xylene and washed with isopropyl alcohol (IPA). Then washed the sections under running tap water for 10 min. Then placed the slides in microwaveable plastic couplin jars filled with 250 mL of 0.01 M citrate buffer (pH - 6) for antigen retrieval. Then heated the jar in pressure cooker for 2 min and placed in coupling jar for 30 min at room temperature. Then washed the sections with PBS for 15 min. Then overlayed the slides with peroxide block solution for 10 min. Washed the sections with PBS (5min x 2). Then incubated the slides with primary antibody (PC-10) for 1 h. Washed the sections with PBS (5 min). Incubated the slides with secondary antibody (Anti-antibody for 30 min). Washed the sections with PBS (5 min). Treated the sections with 3,3 [1] diaminobenzidine tetrahydrochloride (DAB) and buffer substrate for 5-8 min. Then washed with PBS for 5 min. Then washed with tap water. Then counter stained with haematoxylin. Then washed with water and then alcohol and cleared in xylene. Then examined under light microscope (LM). Positive cells were identified by the presence of brickred colour in the nucleus and cytoplasm (Non-mitotic cells- expression of PCNA was restricted to nucleus and in mitotic cells expression was with both nucleus and cytoplasm) and the Ki-67 Index was calculated with the following formula.

$$\text{PCNA Index} = \frac{\text{No. of positive cells/labeled nuclei}}{\text{Total number of cells}} \times 100$$

Results

Out of 21 CMT samples collected 5 (23.80%) were benign and 16 (76.20%) were malignant. The suspected mammary tumours were of the following types. Tubular adenocarcinoma No. 2 (9.52%), Papillary adenocarcinoma No. 3 (14.28%), Papillary cyctic adenocarcinoma No. 1 (4.76%), Solid carcinoma No. 2 (9.52%), Squamous cell carcinoma No. 1 (4.76%), Fibrosarcoma No. 1 (4.76%), Malignant mixed

mammary tumours No. 4 (19.04%), Cavernous haemangioma No.s (9.52%), Fibroadenoma No. 1 (4.76%), Lipoma No.2 (9.52%) and soft tissue hyperplasia No.2 (9.52%).

Proliferating cell nuclear antigen was associated with cell proliferation in different tumours. PCNA positive labeling was observed as brick red to brown coloured nuclei (Fig. 1-3). The mean PCNA positive nuclei in different tumour tissues varied from 3.24±0.06 to 52.35±7.27. The highest index was observed in solid carcinomas (52.35±7.27) and Malignant mixed mammary tumor (50.41±5.26) and the lowest index was observed in soft tissue hyperplasia (3.24±0.06) and lipomas. (4.78±1.46). The mean number of PCNA positive nuclei in benign tumours (5.42±1.48) was significantly (P<0.05) lower than the malignant tumours (39.45±3.84). In the malignant tumours cavernous hemangiomas (20.07±4.23) revealed less PCNA index and solid carcinomas (52.35±7.27) revealed highest PCNA index. While in benign tumours Soft tissue hyperplasia (3.24±0.06) revealed less PCNA index and Fibroadenoma (8.25±1.12 revealed highest PCNA index.

Immuno histochemical staining of Ki-67 antigen gave a mild to strong brown colored nuclear labeling and showed granular, diffuse, nucleolar or mixture of all the types of staining pattern (Fig. 4-6). The mean Ki-67 positive nuclei in different tumour tissues varied from 1.04±0.06 to47.7±5.26. The highest index was observed in Malignant mixed mammary tumor and the lowest index was observed in Soft tissue hyperplasia. The mean number of Ki-67 positive nuclei in benign tumours (6.25±1.51) was significantly (P<0.05) lower than the malignant tumours (32.95±3.16) (Fig. 4.7.2.21). In the malignant tumours Cavernous hemangioma (19.29±1.83) revealed less Ki-67 index and Malignant mixed mammary tumours (47.7±5.26) revealed highest Ki-67 index. While in benign tumours Soft tissue hyperplasia (1.04±0.06) revealed less Ki-67 index and Fibroadenomas (6.25±0.52) revealed highest Ki-67 index. The mean PCNA and Ki-67 Ki-67 index counts were shown in the Table-1. These exist a significant similar correlation between the Ki-67 index and PCNA index with malignancy and poor prognosis.

Table 1: PCNA and Ki-67 counts in different canine mammary tumours

Sl. No.	Type of tumour	No. of cases	PCNA counts 200X Mean±SE	Ki-67 counts 200X Mean±SE
	Malignant Tumours			
1	Tubular adenocarcinoma	2 (9.52%)	38.46±3.56^{a}	29.2±2.32^{a}
2	Papillary adenocarcinoma	3 (14.28%)	46.61±1.89^{b}	38.77±1.09^{c}
3	Papillary cyctic adenocarcinoma	1 (4.76%)	37.25±2.34^{d}	29.23±1.92^{b}
4	Solid carcinoma	2 (9.52%)	52.35±7.27^{c}	39.68±3.56^{e}
5	Squamous cell carcinoma	1 (4.76%)	29.26±1.09^{e}	26±0.92^{d}
6	Fibrosarcoma	1 (4.76%)	41.23±3.26^{g}	33.8±1.35^{h}
7	Malignant mixed mammary tumor	4 (19.04%)	50.41±5.26^{f}	47.7±5.26^{g}
8	Cavernous hemangioma	2 (9.52%)	20.07±4.23^{h}	19.29±1.83^{i}
	Benign tumours			
9	Fibroadenoma	1 (4.76%)	8.25±1.12^{i}	6.25±0.52l^{k}
10	Lipoma	2 (9.52%)	4.78±1.46^{k}	3.14±0.26^{j}
11	Soft tissue hyperplasia	2 (9.52%)	3.24±0.06^{j}	1.04±0.06^{l}

Values are Mean±SE (n=10) of different fields of IHC slides; One-way ANOVA
Means with different superscripts in a column differ significantly at P<0.05 (*)

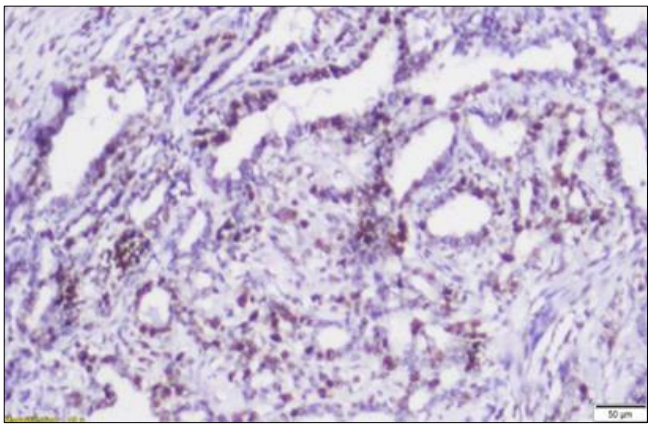

Fig 1: Photomicrograph showing-PCNA immune histochemical staining of tubular adenocarcinoma: ×100.

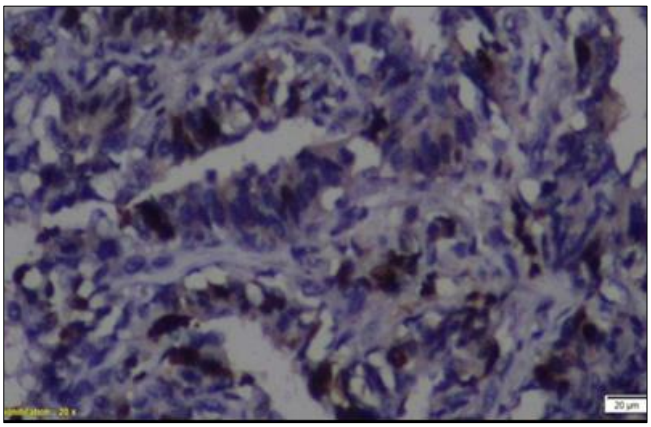

Fig 2: Photomicrograph showing -PCNA immunohistochemical staining of papillary adenocarcinoma: ×200.

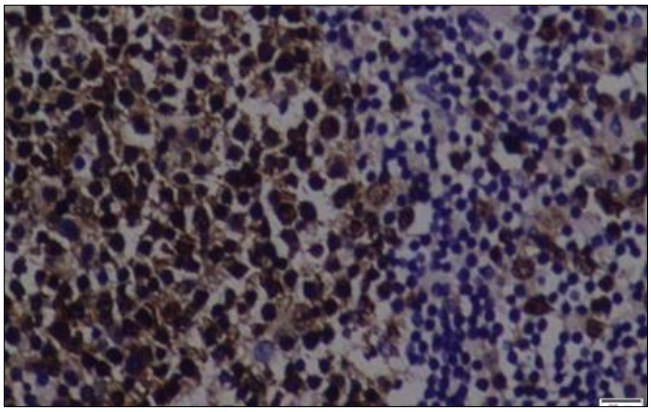

Fig 3: Photomicrograph showing -PCNA immunohistochemical staining of Solid carsinoma: ×200.

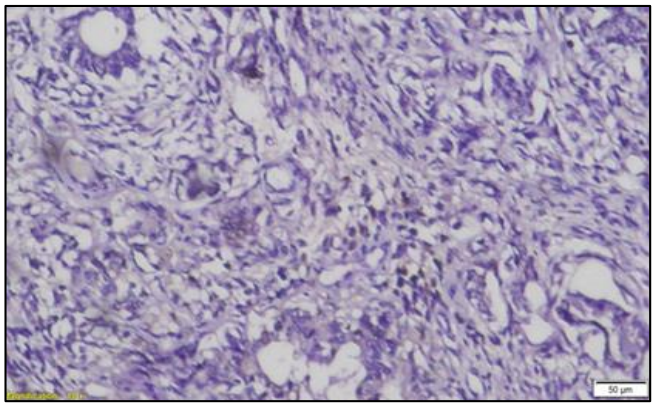

Fig 4: Photomicrograph showing -Ki-67 immunohistochemical staining of Tubular adenocarcinoma: ×100.

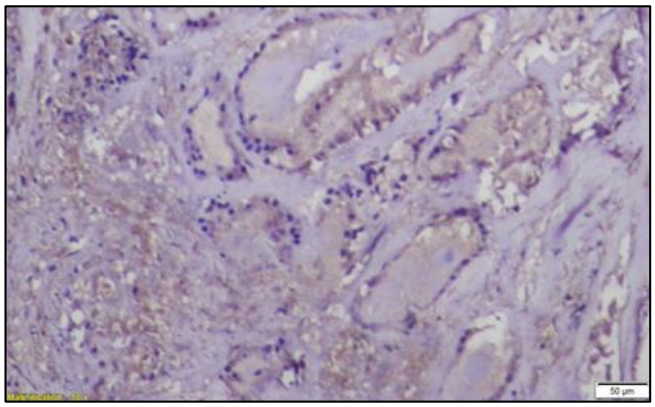

Fig 5: Photomicrograph showing -Ki-67 immunohistochemical staining of Malignant mixed mammary tumour: ×100.

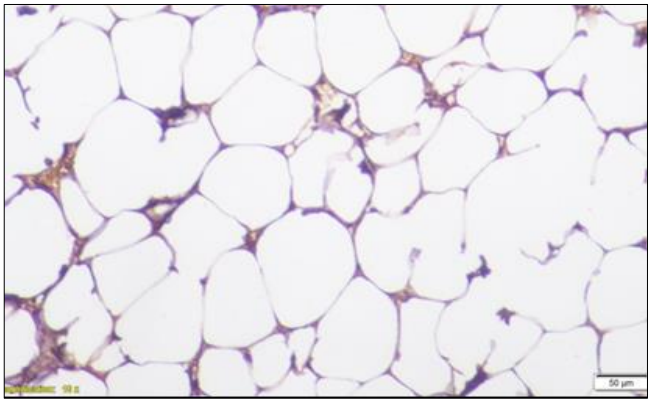

Fig 6: Photomicrograph showing-Ki-67 immuno histochemical staining of Lipoma: ×100.

Discussion

Proliferating cell nuclear antigen is a 36 KD acidic auxiliary nuclear protein of DNA polymerase expressed in dividing cells during G_1 phase of cell division. PCNA increases in late G_1 peaks in S-phase and declines during G_2 and M phase. Its levels correlates directly with rates of cellular proliferation and DNA synthesis. PCNA may act as auxiliary protein of DNA polymerase delta to play a fundamental role in initiation of cell proliferation. Two population of PCNA are seen. The first type is expressed exclusively during S-phase and the second type is expressed in a non-specific, mainly associated with cytoskeletal elements during metaphase (Sivaseelan 2018).

In the present study the mean values of PCNA positive nuclei in different tumour tissues varied from 3.24±0.06 to 52.35±7.27. The mean number of PCNA positive nuclei in malignant tumours were significantly higher than benign tumours. The observations were in accordance with Preziosi *et al.* (1995) [12], Ilhan *et al.* (2008) [13], Chandravathi 2008) [8], Carlho *et al.* (2016) [14]. Ranganath *et al.* (2011) [15] observed that the reactivity to PCNA was localized to nuclei in non mitotic cells and in miotic cells PCNA immunostaining was cytoplasmic.

Ki-67 is a nuclear protein expressed during all active stages of cell cycle (G, S, G_2 and Mphase) but absent in resting, non-cycling cells (G_0). It is a chromosomal DNA matrix and possibly play a role in breakdown of nuclear envelope during cell division. Ki-67 expressed 24-26 h after cell stimulation in the G_1 phase and remains immunohistochemically detectable throughout the interphase of the cell cycle, reaching its maximum level during mitosis (Sivaseelan 2018) [5].

In the present study the mean Ki-67positive nuclei in different tumours ranged from 1.04±0.06 to47.7±5.26. The mean number of Ki-67 positive nuclei in benign tumours was significantly (P<0.05) lower than the malignant tumours. The observations were in accordance with Geraldes *et al.* (2000) [16], Alenza *et al.* (2004) [17], Kadthur *et al.* (2007) [18], Ferreria *et al.* (2009) [19]. Ranganath *et al.* (2011) [15] observed that the reactivity of Ki-67 was localized to nuclei in non mitotic cells and in mitotic cells Ki-67 expression was chromosomal. In conclusion, higher PCNA and Ki-67 counts in the tumours were associated with malignancies and poor prognosis.

References

1. Hellmen I, Cornelisse CJ, Van Den Burgh BART. PS3 mutations in mammary tumor cell lines and corresponding tumor tissues in the dog. Anticancer Research. 1996;16:3737-3744.
2. Benjamin SA, Lee AC, Saunders WJ. Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles. Veterinary Patholog. 1999;36(5):423-436.
3. Hampe JF, Misdorp W. Tumours and dysplasias of the mammary gland. Bulletin of the World Health Organization. 1974;50(1-2):111.
4. Hellmen E, Bergstrom R, Holmberg L, Spangberg IB, Hansson K, Lindgren A. Prognostic factors in canine mammary tumors: A multivariate study of 202 consecutive cases. Veterinary Pathology 1993;30(1):20-27.
5. Sivaseelan S. Animal Oncology. Edn1, Vol.1, Associated publishing company, New Delhi, 2021.
6. Gilbertson SR, Kurzman ID, Zachrau RE, Hurvitz AI, Black MM. Canine mammary epithelial neoplasms:

biologic implications of morphologic characteristics assessed in 232 dogs. Veterinary Pathology. 1983;20(2):127-42.
7. Henry CJ. Biomarkers in veterinary cancer screening: applications, limitations and expectations. The Veterinary Journal. 2010;185(1):10-4.
8. Chandravathi T. Pathology of neoplasms in dogs (Accession No. 5810081166) [M.V.Sc. dissertation, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India]. Krishikosh Dissertations publishing, 2010.
9. Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Krol M and Jurka P. Current biomarkers of canine mammary tumors. Acta Veterinaria Scandinavica. 2018;60(1):1-13.
10. Duffy MJ. Use of biomarkers in screening for cancer. Advances in Experimental Medicine and Biology 2015;867:27-39.
11. Luna LG. Manual of histologic staining methods of the Armed Forces institute of Pathology, 3rd edition. Mc. Graw Hill Book Co., New York, 1968.
12. Preziosi R, Sarli G, Benazzi C, Marcato PS. Detection of proliferating cell nuclear antigen (PCNA) in canine and feline mammary tumours. Journal of Comparative Pathology. 1995;113(4):301-13.
13. Ilhan F, Metin N, Birincioglu ST. Immunohistochemical detection of PCNA and p53 in mammary tumours and normal tissues in dogs. Revue de Medecine veterinaire. 2008;159(5):298.
14. Carvalho MI, Pires I, Prada J, Lobo L, Queiroga FL. Ki-67 and PCNA expression in canine mammary tumors and adjacent nonneoplastic mammary glands: prognostic impact by a multivariate survival analysis. Veterinary Pathology. 2016;53(6):1138-46.
15. Ranganath GJ, Kumar R, Reddy AP, Pawaiya RV, Maiti SK. Comparative study on the expression pattern of the proliferating cell markers PCNA and Ki67 in canine mammary tumours. Indian journal of Veterinary Pathology. 2011;35(1):13-17.
16. Geraldes M, Gärtner F, Schmitt F. Immunohistochemical study of hormonal receptors and cell proliferation in normal canine mammary glands and spontaneous mammary tumours. Veterinary Record 2000;146(14):403-6.
17. Alenza MP, Pena L, Castillo ND, Nieto AI. Factors influencing the incidence and prognosis of canine mammary tumours. Journal of Small Animal Practice 2000; 41(7):287-91.
18. Kadthur JC, Rao S, Sonnahallipura BM, Thimmanahalli DS, Laxmikanth SM. Prognostic value of Ki 67 proliferation antigen in canine malignant mammary gland tumours. Brazilian Journal of Veterinary Pathology. 2011;4(1):36-40.
19. Ferreira E, Bertagnolli AC, Cavalcanti MF, Schmitt FC, Cassali GD. The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours. Veterinary and Comparative Oncology. 2009;7(4):230-5.
20. Abdelmegeed SM, Mohammed S. Canine mammary tumors as a model for human disease. Oncology letters. 2018;15(6):8195-205.