Clinics in Oncology

Research Article
Published: 13 May, 2023

Immunohistochemical Detection of Prognostic Biomarkers in Canine Mammary Tumors and Relation with Malignancy and Prognosis

Kavisha G[1], Neeraj K[1], Avantika S[1], Motilal BA[1], Abhay BY[1], Suresh PC[1], Himani P[1], Prabhu SN[1], Renu S[1], Soumen C[2] and Sanjay P[3]*

[1]Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Veterinary University, India

[2]Department of Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Veterinary University, India

[3]Department of Surgery and Radiology, College of Veterinary Science and Animal Husbandry, Veterinary University, India

Abstract

A definitive and early diagnosis is essential in neoplastic cases either in case of humans or animals for determining the line of treatment and better prognosis. The present study was conducted to record the prognostic biomarkers (Estrogen Receptor Alpha- (ERα), Progesterone Receptor- (PR), Human Epidermal growth factor Receptor 2- (HER-2) and Protein-53 (P-53) of different types of mammary gland tumors occurring in canines based on histopathological and immunohistochemical examination study of prognostic biomarkers (ERα, PR, HER-2 and P-53). Total 53 cases suspected for Canine Mammary Tumor (CMT) were encountered at Veterinary Clinical Complex, DUVASU, Mathura, India during the period from January 2021 to April 2022. Out of them 42 were neoplastic and 11 were non-neoplastic cases. The 2 cases out of total 42 neoplastic cases showed distant metastases in lungs. Out of 42 cases, total 24 tissue samples were obtained for further examination. Histopathological examination showed that more than one histopathological type of mammary tumor was present in one case, so total number of 32 tumors was found in 24 tissue samples. Most of the cases were malignant neoplasms with maximum occurrence of carcinoma solid (12.5%) and tubulo-papillary carcinoma (12.5%). Immunohistochemical examination showed that most of the malignant mammary neoplasm cases were negative for ERα, PR, HER-2 expressions i.e., triple negative and positive for P-53 immunohistochemistry expression 8/24 or 33.34% showed poor prognosis. The expression of biomarkers analysis and interpretation provides valuable diagnostic and prognostic significance regarding nature of tumor and start of treatment regimen in canine mammary tumors.

Keywords: Canine Mammary Tumor; Biomarkers; Immunohistochemistry; Prognosis

OPEN ACCESS

Correspondence:
Neeraj Kumar Gangwar, Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Veterinary University, Mathura, Uttar Pradesh, 281001, India, E-mail: neerajgangwarvet@gmail.com

Received Date: *23 Apr 2023*
Accepted Date: *08 May 2023*
Published Date: *13 May 2023*

Citation:
Kavisha G, Neeraj K, Avantika S, Motilal BA, Abhay BY, Suresh PC, et al. Immunohistochemical Detection of Prognostic Biomarkers in Canine Mammary Tumors and Relation with Malignancy and Prognosis. Clin Oncol. 2023; 8: 1999.

ISSN: 2474-1663

Introduction

Cancer is among one of the most leading causes of death worldwide according to World Health Organization. In human female breast cancer is most diagnosed and related with cancer associated mortality in females [1]. Mammary tumors in case of dogs are the second most common neoplasm after skin tumors [2]. About 52% of all tumor cases in dogs are mammary tumors [3]. As there are clinical and molecular similarities between human breast cancer and canine mammary tumors along with physiological similarities between both human and canines, the canines act as an excellent model for human breast cancer studies [4]. The biological behavior of these tumors showed wide variation in morphological characteristics so determination of histological type and grade of malignancy is very important for clinicians to determine the prognosis of the condition [5]. The results of histopathological studies provide an insight into development of canine mammary tumors. Histopathology is subjective and may not always predict the clinical nature. Hence, in present scenario interest has been developed in measuring the non-subjective biological parameters such as tumor markers often called as biomarkers [6]. With the help of immunohistochemistry technique, the diagnosis and therapeutic targets could be identified, which would ultimately lead to improvement in overall survival of the patients [7].

Material and Methods

Samples for prospective study were collected during the period from January 2021 to April 2022 from Veterinary Clinical Complex, DUVASU, Mathura, UP, India. Total 53 dogs suspected for canine mammary tumors were presented during the study period and out of which 42 dogs were showing neoplastic condition, while in 11 dogs, the mammary glands showed non-neoplastic growth, when examined with ultrasonographic and Fine Needle Aspiration Cytology. The study was preceded with the approval of Institutional Animal Ethics Committee with application number IAEC/22/22 dated- 31/01/2021. Total 24 tissue samples were obtained from 42 cases.

Collection of tissue samples for histopathological examination and immunohistochemistry

Samples were collected from animals during surgery under general anesthesia. Tissue sample of about 2 cm to 4 cm from different locations of excised tumor tissue was collected in 10% neutral buffered formalin. The control tissues (5 samples) were taken from healthy animal with the help of punch biopsy under local anesthesia with the consent of pet owner.

Histopathological analysis

This technique plays a key role in diagnosis and acts as gold standard for determining the changes in tissue. Similarly in tumor condition it plays a vital role in identification of tumor type, grade of malignancy and characterizing various changes occurring in tissue in tumor condition. The tissues were properly processed by standardized processing protocol and stained with standard routine Hematoxylin and Eosin staining for histopathological analysis [8,9]. The histopathological sections were analyzed after routine H and E staining of tissue sections and classified according to Goldschmidt et al. [10], classification criterion and histopathological grading of CMTs based on Elston and Ellis system of classification [11].

Immunohistochemistry analysis

Immunohistochemistry was done to determine the degree of expression of mammary tumor biomarkers to classify the different samples into benign and malignant [12]. The tissues were taken on APES coated slides and further deparaffinized at 62°C for 25 min in hot air oven. Slides were then cleared and rehydrated by putting them in xylene (2 changes) and descending grades of alcohol for 5 min each respectively. Further slides were washed with phosphate buffered solution with pH 7.4 (3 changes) for 10 min each. The antigen retrieval was done by keeping the slides in sodium citrate antigen retrieval solution (pH- 6.0) by pressure cooker method. Slides were then incubated with 3% H_2O_2 for 15 min to 20 min for blocking of endogenous peroxidase and after it the slides were incubated with 3% bovine serum albumin for blocking of non-specific binding. Further the slides were incubated with primary antibody procured from Invitrogen (rabbit anti-estrogen receptor-alpha monoclonal antibody 1:50 dilution, rabbit anti-progesterone monoclonal antibody 1:100 dilution, mouse HER-2 monoclonal antibody 1:40 dilution, mouse P53 monoclonal antibody 1:30 dilution) for overnight at 4°C in a moist chamber. In negative control tissue sections instead of primary antibody PBS was used. Sections were then washed with PBS two changes for 5 min each. The slides were then incubated with secondary antibody, 1:100 dilution (Invitrogen – PA1-28567 Rabbit anti-mouse biotinylated secondary antibody for HER-2 and P-53 and immuno-Cruz rabbit ABC staining system sc-2018 kit - mouse anti-rabbit secondary antibody for Estrogen receptor alpha and Progesterone receptor) for 1 h at room temperature. Slides were then washed with phosphate buffered saline (2 changes) for 10 min each and sections with HER-2 and P-53 were incubated with avidin peroxidase with 1:100 dilution for 30 min. Slides were washed in PBS with two changes each for 5 min and properly wiped with tissue paper. DAB (pH 7.2) working solution was poured onto the slides over tissue sections and kept for 5 min. The reaction was checked by keeping the slides in tap water. Counterstaining was done with Harris hematoxylin for 45 sec and then the slides were rinsed with deionized water. Tissue sections were dehydrated by passing the slides through ascending grades of alcohol (50% to 100%) for 2 min each and cleared by putting in xylene for 5 min with two changes and finally mounted with DPX to observe under microscope. Immunohistochemistry scoring of ERα, PR was done according to Allred Scoring System [13]. The percentage of ERα- or PR-positive cells was calculated by counting ≥ 1000 cells within 10 distinct, randomly selected microscopic fields (400x) [14]. Scoring of HER-2 was done according to Pena et al. [13]. The internationally accepted system for HER-2 IHC scoring is HercepTest Scoring and according to it both +2 and +3 should be considered as positive. Scoring for P-53 was done according to Pena et al. [15]. P-53 is considered positive only if >5% cells are positive [13].

Statistical analysis

Fisher's exact test and Chi square test was run to find the association between ERα, HER-2, and PR expression in benign and malignant tumors.

Results

In current study most of the cases were encountered in intact bitches of more than 7 years age. The purebred dogs were more affected as compared to crossbred and among them highest occurrence was observed in German shepherd breed of dogs (28.57%). The posterior mammary gland showed higher occurrence as compared to anterior mammary glands and among them the inguinal mammary glands showed highest occurrence (35.29%).

Table 1: Classification of different tumors and their percentage in the current study (n=32).

S. No.	Type of tumour	Number	Percentage (%)
1	Carcinoma solid	4	12.5
2	Tubulo-papillary carcinoma	4	12.5
3	Ductal carcinoma	3	9.375
4	Fibroadenoma	3	9.375
5	Intraductal papillary carcinoma	2	6.25
6	Carcinoma in-situ	2	6.25
7	Carcinoma mixed type	2	6.25
8	Benign mixed tumour	2	6.25
9	Malignant myoepithelioma	2	6.25
10	Complex adenoma	1	3.125
11	Complex carcinoma	1	3.125
12	Tubular carcinoma	1	3.125
13	Tubular carcinoma (cystic type)	1	3.125
14	Carcinoma spindle cell variant	1	3.125
15	Comedocarcinoma	1	3.125
16	Fibrosarcoma	1	3.125
17	Squamous cell carcinoma	1	3.125
Total		**32**	**100**

Table 2: Classification of tumor based on malignancy (n=32).

Type of tumour	Number of tumours	Percentage (%)
Benign	6	18.75%
malignant	26	81.25%
Total	**32**	**100%**

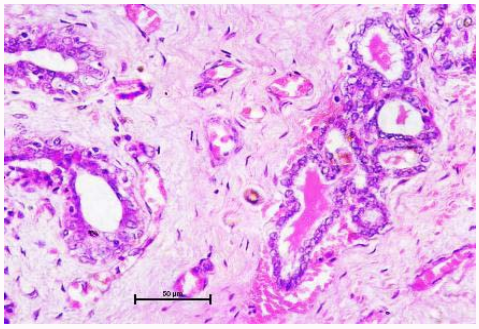

Figure 1: Normal mammary gland tissue containing lobules which had most of the inactive alveoli without secretions and some active alveoli with secretions. There was presence of abundant interlobular as well as intralobular connective tissue stroma supporting the lobules and alveoli. H&E 400x.

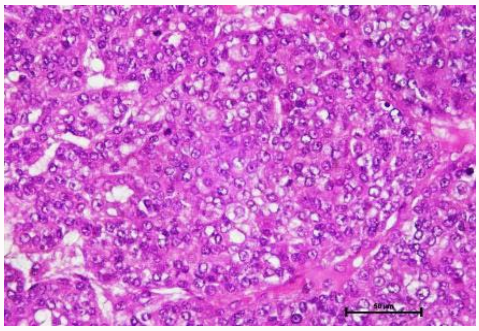

Figure 2: Carcinoma solid: The cells are closely packed and arranged in the form of solid masses without lumina. The closely packed solid masses of cells form dense, irregular sized lobules which were supported by fine fibrovascular stroma. H&E 400x.

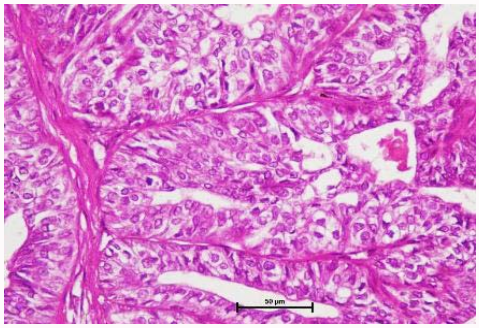

Figure 3: Tubulo-papillary carcinoma: The neoplastic tubules were mainly arranged in pedunculated papillary manner. The papillae were supported by fine fibrovascular connective tissue and extended into the tubular lumina. The cells were arranged in stratified manner (2-3 cells thick) lining the tubules. H&E 400x.

Histopathological examination of tissue samples: Out of 42 neoplastic cases, 24 tissue samples were collected (including sample of lung nodule in metastatic case). Healthy mammary gland tissue was taken as positive control. Out of 24 tissue samples, 17 different

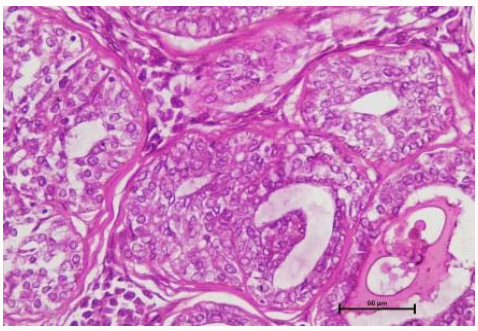

Figure 4: Ductal carcinoma: Differentiation of neoplasm into interlobular ducts. The neoplastic cells were arranged in tubules and cords which surrounded slit like lumina. The lumina lined by double layer of cuboidal to columnar epithelial neoplastic cells. H&E 400x.

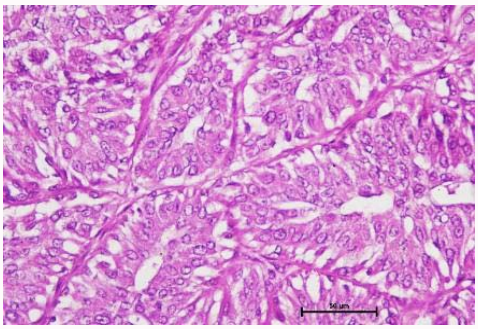

Figure 5: Intraductal papillary carcinoma: The epithelial cells proliferated in stratified fashion (cells were arranged in multi-layered manner). Fibrous connective tissue and myoepithelial cells were present which acted as supporting stroma for papillae.

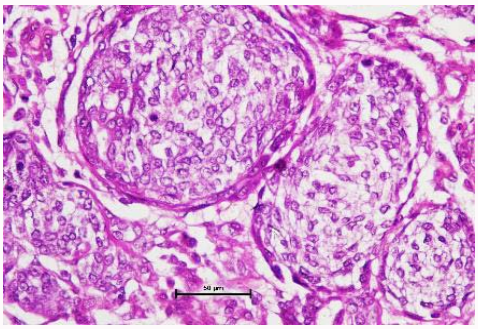

Figure 6: Carcinoma *in-situ*: Consisted of densely cellular well demarcated nodules which have not extended through the basement membrane into surrounding mammary tissue. The cells are polygonal to round and closely packed in the nodules H&E 400x.

histopathological types of tumors were identified. In some cases, more than one histological type of tumor was found in same case, so total number of 32 tumors was found in 24 cases (Table 1). In present study, the most common type of malignant mammary tumors found were carcinoma solid and tubulopapillary carcinoma while most common type of benign tumor found was fibroadenoma (Table 2 and Figures 1-18).

Total 27 samples were graded out of 32 tumors (Table 3), as tumor of mesenchymal origin were excluded from grading because the grading system of Pena et al. [11], was not applicable on mesenchymal tumors (Fibroadenoma, complex adenoma and fibrosarcoma were excluded).

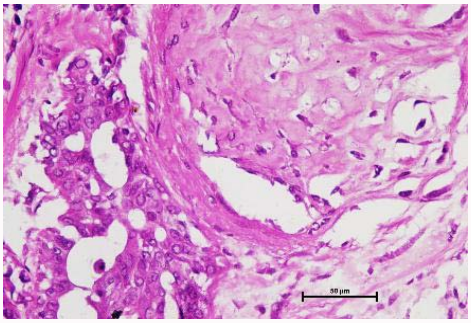

Figure 7: Carcinoma mixed type: Presence of three or more cell population supported by fibrovascular stroma. The first population formed by cells arranged in irregular tubules, second population formed by spindle shaped myoepithelial cells and the third population formed by foci of cartilage. H&E 400x.

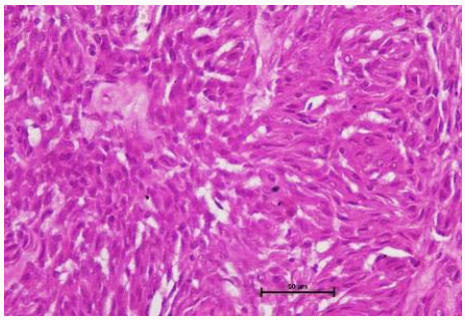

Figure 8: Malignant myoepithelioma: Characterized by two cell population that were epithelial and myoepithelial components and both were malignant, supported by moderate amount of fibrous tissue stroma. H&E 400x.

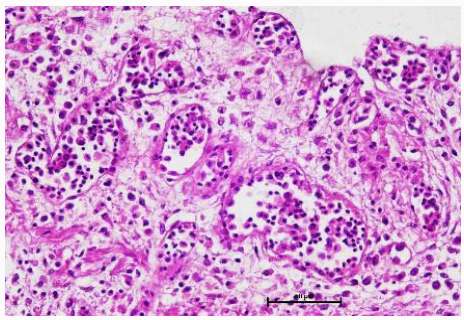

Figure 9: Complex carcinoma: Presence of malignant epithelial and benign myoepithelial component. Focal necrosis was present in few epithelial cells with squamous differentiation at some places. Presence of severe lymphatic invasion by the neoplastic cells. H&E 400x.

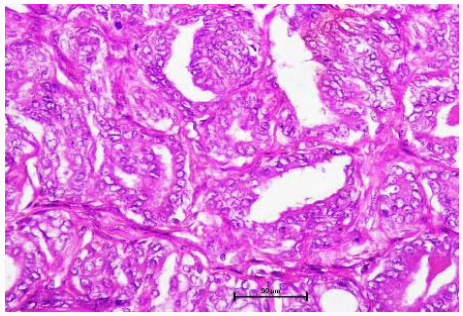

Figure 10: Tubular carcinoma: Cells mainly arranged in a tubular fashion. Lining of tubules is 1-2 cells thick and extensive proliferation of myofibroblasts at few places. H&E 400x.

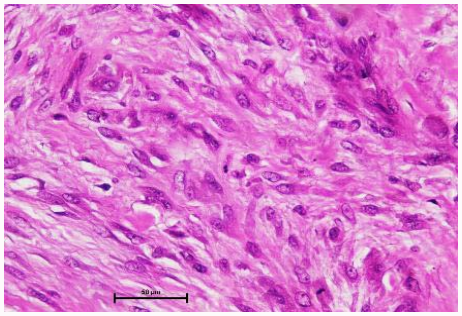

Figure 11: Carcinoma spindle cell variant: Presence of islands and cords of epithelial cells which are supported by fine fibrous stroma. The cells as well as nuclei are large and fusiform. At some places foci of squamous epithelial differentiation present. H&E 400x.

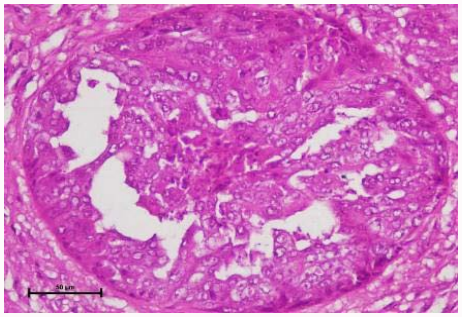

Figure 12: Comedocarcinoma: Presence of neoplastic cell aggregates containing necrotic areas within the center. In these necrotic areas there are abundant eosinophilic material admixed with necrotic neutrophils and cell debris. The neoplastic cells are closely packed at the periphery and supported by fine fibrovascular connective tissue stroma. H&E 400x.

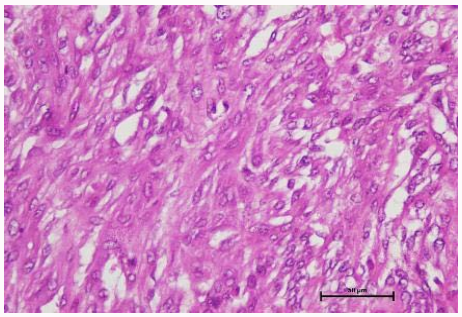

Figure 13: Fibrosarcoma: The fusiform cells proliferated in a distinct interwoven pattern. The cells were having indistinct cell borders and large oval to elongated nuclei. H&E 400x.

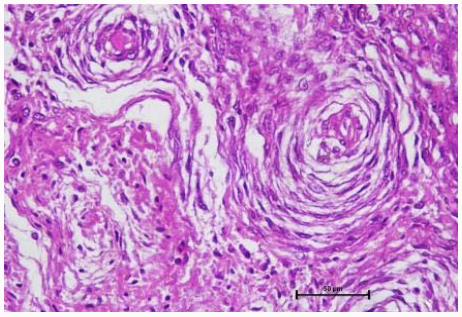

Figure 14: Squamous cell carcinoma: This neoplasm solely consisted of squamous epithelium. Presence of islands and cords of epithelial cells which formed the keratin pearls. The cells and nuclei are large. Numerous mitotic figures are present. H&E 400x.

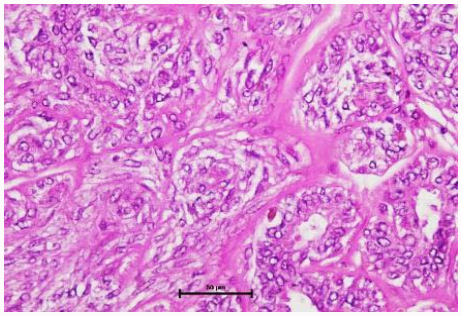

Figure 15: Fibroadenoma: The neoplastic masses consist of tubules lined by cuboidal to columnar cells that has round and uniform nuclei. The tubules are surrounded by extensive stroma of loose fibrous connective tissue. H&E 400x.

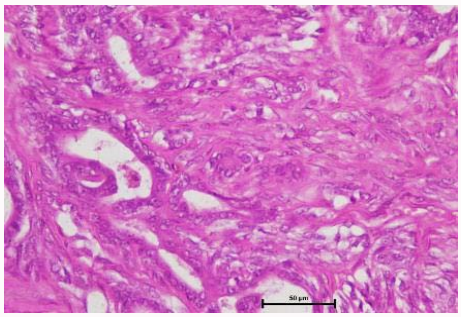

Figure 16: Complex adenoma: Composed of both epithelial and myoepithelial cell population with variable amount of fibrous stroma. Cuboidal to columnar epithelial cells lining the tubules. Myoepithelial cells are spindle to stellate shaped and abundant fibrillar myxoid matrix was present in association with these cells. H&E 400x.

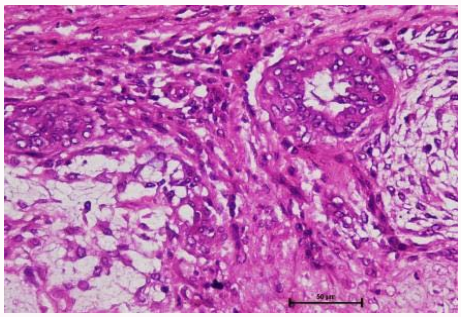

Figure 17: Benign mixed tumor: Both epithelial and myoepithelial cell population with foci of cartilage or bone and variable amount of fibrous stroma. Cuboidal to columnar epithelial cells lining the tubules. Myoepithelial cells admixed with fibrillar matrix and multifocally there are areas of cartilage. H&E 400x.

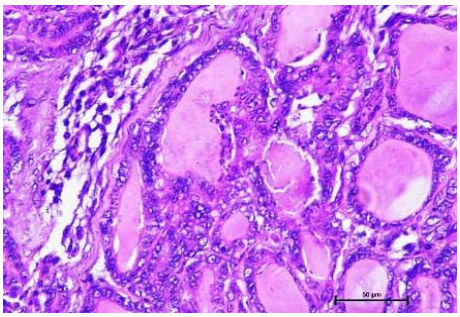

Figure 18: Tubular carcinoma cystic type: In case of lung metastases tissue sections showed characters similar to tubular carcinoma with dilated cystic lumina of tubules filled with eosinophilic secretion. H&E 400x.

Table 3: Grading of tumor based on histopathological examination according to Elston and Ellis grading system [11] (n=27).

Grade of tumour	Number	Percentage
Grade I	6	22.22%
Grade II	18	66.67%
Grade III	3	11.11%
Total	**27**	**100%**

Table 4: Percentage of different IHC expression of samples (n=24).

ERα	PR	HER-2	P53	Total	Percentage (%)
-	-	-	+	8	33.34
-	-	+	+	6	25
+	+	-	-	3	12.5
-	+	+	+	1	4.17
+	-	-	-	1	4.17
+	+	+	+	1	4.17
-	-	-	-	1	4.17
+	-	+	+	1	4.17
-	+	+	-	1	4.17
+	-	-	+	1	4.17

Table 5: Percentage of IHC expression of each marker.

Marker	Expression	Number	Percentage (%)
ERα	+	7	29.17
	-	17	70.83
PR	+	6	25
	-	18	75
HER-2	+	10	41.67
	-	14	58.33
P-53	+	18	75
	-	6	25

Table 6: Association of HER-2 with Erα and PR in benign cases.

		HER-2 Negative	HER-2 Positive	P value
1. ERα	Negative	0	1 (16.7%)	p>0.05
	Positive	4 (66.6%)	1 (16.7%)	
2. PR	Negative	1 (16.7 %)	0	p>0.05
	Positive	3 (50%)	2 (33.3%)	

Table 7: Association of HER-2 with Erα and PR in malignant cases.

		HER-2 Negative	HER-2 Positive	P value
1. Erα	Negative	9 (50%)	7 (39%)	p>0.05
	Positive	1 (5.5%)	1 (5.5%)	
2. PR	Negative	10 (55.5%)	7 (39%)	p>0.05
	Positive	0	1 (5.5)	

Immunohistochemistry examination of tissue samples: Out of 42 neoplastic cases, 24 tissue samples were collected, similar to the tissue samples collected for histopathological examination. Healthy mammary gland tissue was taken as positive control. Most of the cases were malignant with triple negative (ERα, PR, HER-2 negative) and positive p-53 immunohistochemistry expression (8/24 or 33.34 %), while in one malignant case the expression was triple negative with negative P-53 (1/24 or 4.17%) (Table 4, 5 and Figures 19-36).

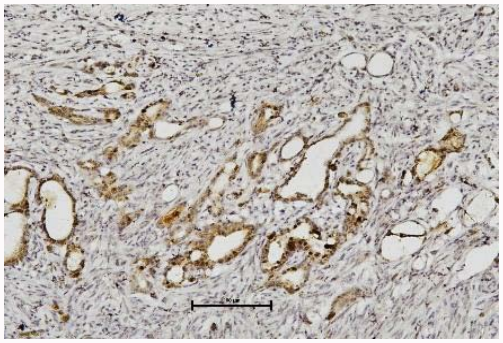

Figure 19: Positive control ERα showing staining in duct epithelial cells. IHC IPOx200x.

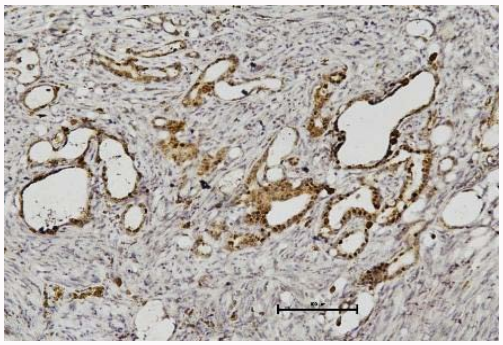

Figure 20: Positive control PR showing staining in duct epithelial cells. IHC IPOx200x.

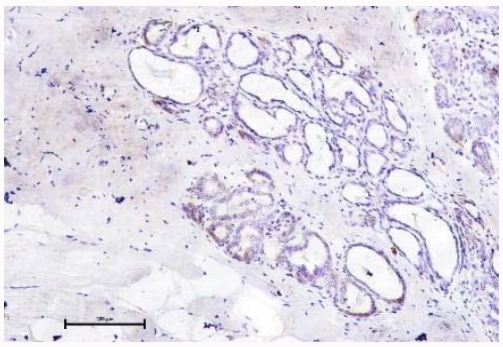

Figure 21: Positive control HER-2 showing no immunoreactivity. IHC 200x.

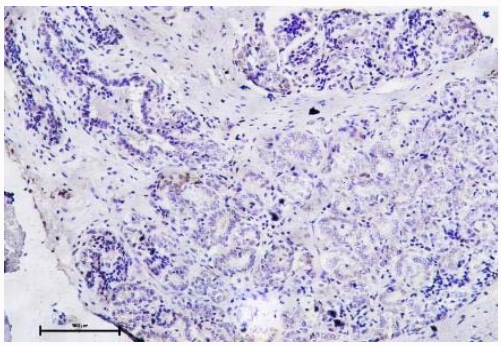

Figure 22: Positive control P-53 showing no immunoreactivity. IHC IPOx200x.

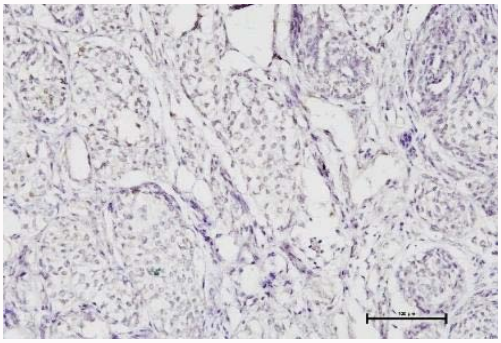

Figure 23: Negative control ERα. IHC 200x.

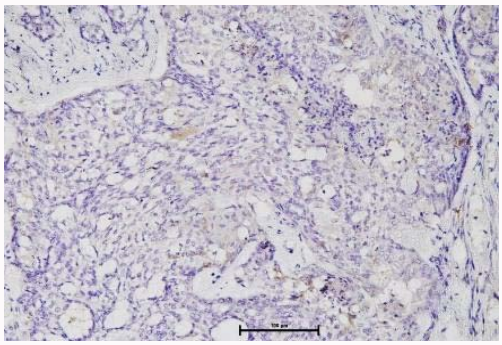

Figure 24: Negative control PR. IHC 200x.

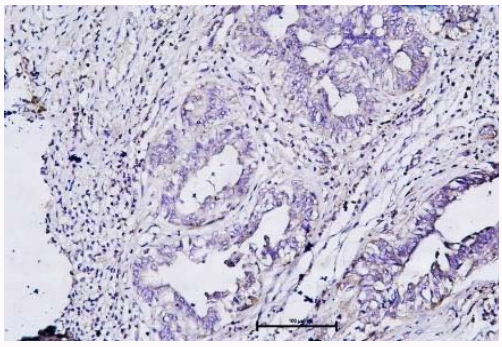

Figure 25: Negative control HER-2. IHC 200x.

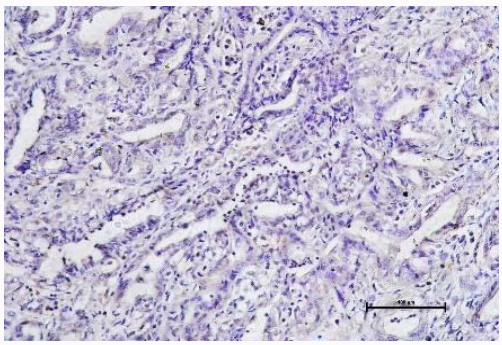

Figure 26: Negative control P-53. IHC IPOx200x.

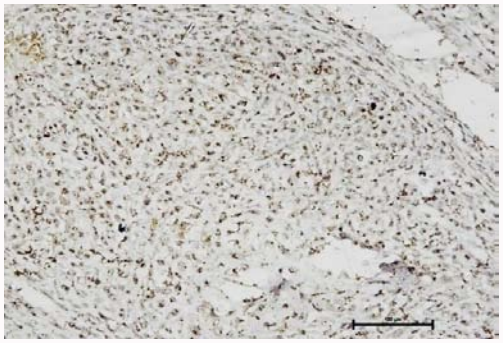

Figure 27: IHC expression of ER showing about 30% positive cells (3) and nuclear staining of moderate intensity (2) (Total score = 5) representing ER +2 expression. ER- Positive. IPOx200x.

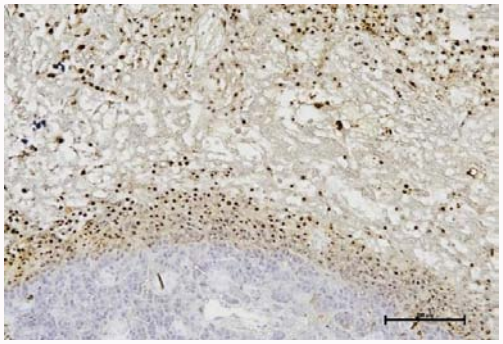

Figure 28: IHC expression of ER showing >50% positive cells (4) and nuclear staining of strong intensity (3) (Total score = 7) representing ER +3 expression. ER- Positive. IPOx200x.

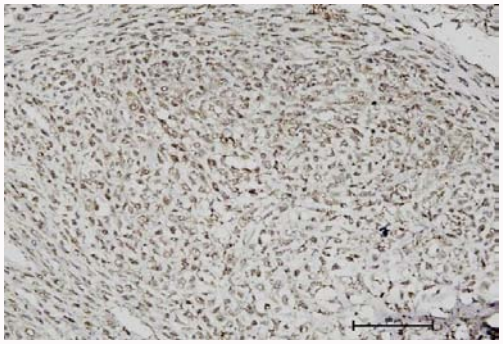

Figure 29: IHC expression of PR showing about 50% positive cells (4) and nuclear staining of moderate intensity (2) (Total score = 6) representing PR +2 expression. PR- POSITIVE. 200x.

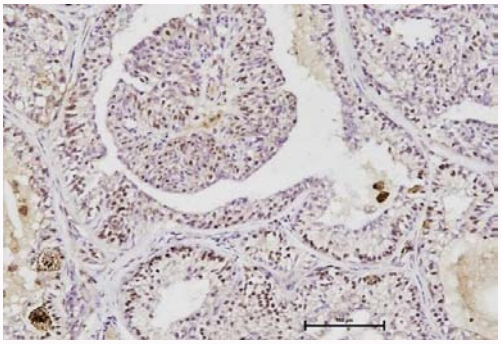

Figure 30: IHC expression of PR showing >70% positive cells (5) and nuclear staining of strong intensity (3) (Total score = 8) representing PR +3 expression. PR- Positive. IPOx200x.

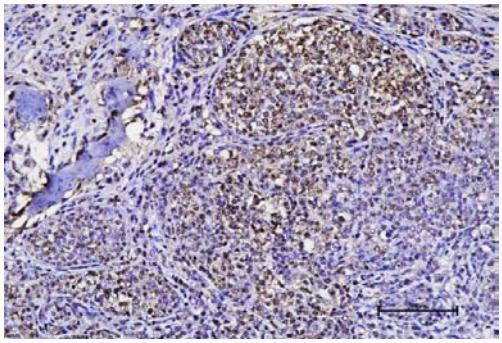

Figure 31: IHC expression of HER-2 showing weak membrane labelling in about 20% of cells representing HER-2 +1 expression. 200x.

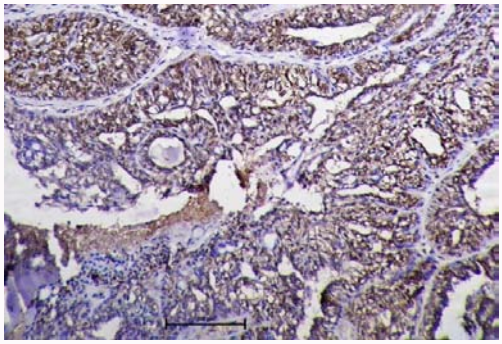

Figure 32: IHC expression of HER-2 showing moderate heterogenous complete membrane labelling in about 50% of cells representing HER-2 +2 expression. IPOx200x.

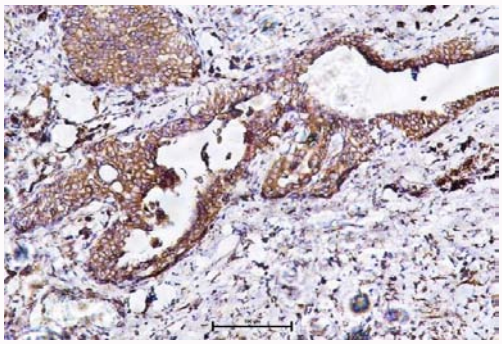

Figure 33: IHC expression of HER-2 showing strong and complete membrane labelling in >50% of cells representing HER-2 +3 expression.

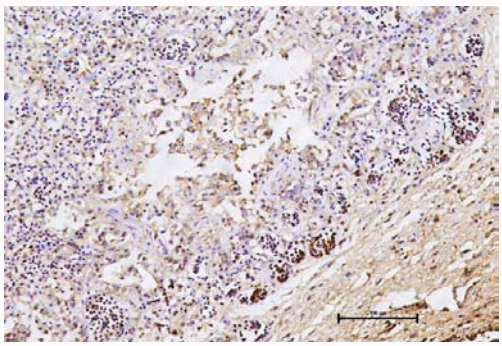

Figure 34: IHC expression of P-53 showing strong labelling in about 15% of tumor cells (score 2). P-53 Positive. IPOx200x.

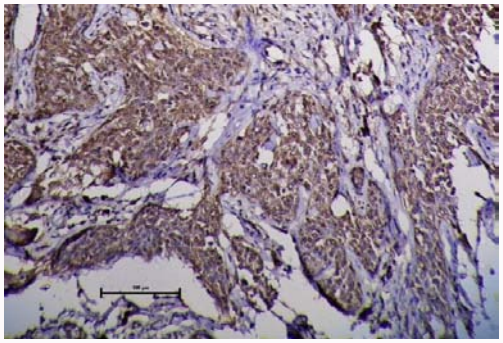

Figure 35: IHC expression of P-53 showing strong labelling in about 40% of tumor cells (score 3). P-53 POSITIVE. 200x.

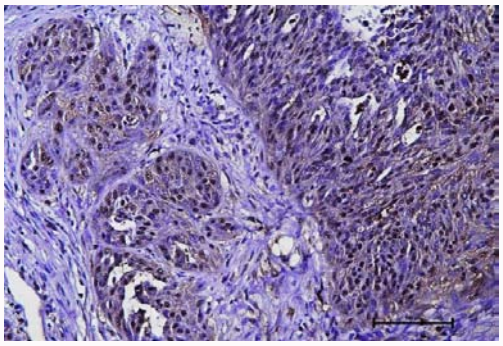

Figure 36: IHC expression of P-53 showing strong labelling in >70% of tumor cells (score 4). P-53 Positive. IPOx200x.

There is no statically significant association (p>0.05) observed in any pair. However, individually these markers play an important role in determining the malignancy of the tumor (Table 6, 7).

Discussion

The results of histopathological studies provide an insight into development of canine mammary tumors. In current findings it was observed that tumors of larger size were mostly more malignant as compared to that of smaller size which might be due to higher growth rate and proliferating potential of malignant tumors [16].

In current findings most of the cases were malignant 81.25% (26/32) while 18.75% (6/32) cases were benign which showed correlation with previous studies [17-19]. Among malignant tumors carcinoma solid (12.5%) and tubulopapillary carcinoma (12.5%) showed maximum occurrence followed by ductal carcinoma (9.375%) [20]. Among benign mammary tumors fibroadenoma (9.375%) showed maximum occurrence followed by benign mixed tumor (6.25%) [17-19]. Histopathological grading of canine mammary tumors provides potential information regarding prognosis of CMTs.

The immunohistochemical examination played an important role in diagnosis and identification of neoplasm in both human and veterinary practices. With the help of this technique therapeutic targets could be identified which would ultimately lead to improvement in overall survival of patients [7]. The findings of the study revealed that the expression of ER and PR was higher in benign tumors while ER expression was lower to absent in malignant tumors. However, PR expression can be seen in intermediate stages in malignant tumors [15,21]. The expression of these hormone receptors aids in differentiation of the neoplasm, thereby aiding assessment of patient prognosis [22]. Immunoreactivity of hormone receptor was observed in the nucleus of neoplastic cells but sometimes cytoplasm may also show reactivity. The immunoreactivity of ER and PR is either low or absent in malignant tumors and associated with worse prognosis while higher immunoreactivity is seen in benign tumors [23,24].

The immunohistochemical expression of HER-2 was seen mainly in membrane and in nuclei of the cells. In current study, the higher expression of HER-2 was seen in malignant tumors which were associated with the poor prognosis [25,26]. HER-2 expression in association with ER and PR expression was found to be more reliable in evaluating the prognosis of the patient in current investigation [21] Protein P-53 showed immunoreactivity in cytoplasm as well as nuclei of neoplastic cells with positive expression in 75% of tumors out of which higher expression was observed in malignant tumors (94.44%) while few benign tumors (5.56%) also showed positive expression and related to poor prognosis in present findings [27,28].

In present findings most of the cases were malignant based histopathological findings which was highly correlated with the immunohistochemistry findings having the expression as ERα-/PR-/HER2-/P53+ which was also associated with poor prognosis of cases [21,29-31]. Though the expression of HER-2 increases with malignancy but the triple negative expression (ERα-/PR-/HER2-) is related with worst prognosis of the case however, the reason behind this finding could not be explained clearly and further researches are going on to attain promising mechanism behind it [21,31].

Conclusion

According to the current findings the tumors with immunohistochemical expression ERα-/PR-/HER 2-/P-53+ were malignant also in histopathological examination which showed higher correlation between these techniques in evaluating the accurate diagnosis of condition. The positive expression of all three receptors showed good prognosis as compared to triple negative (ERα-/PR-/HER 2-) canine mammary tumors showed poor prognosis and high malignancy.

Acknowledgement

Kavisha Gangwar and Neeraj Kumar Gangwar designed plan of study, carried out histopathological and immunohistochemical examination, Avantika Srivastav, Barbaile Ashvin Motilal, Bhate Yash Abhay Prajwal Suresh Chaple and Himani Pandey contributed in sample collection, Renu Singh, Shyama Narayan Prabhu, Soumen Chaudhary and Sanjay Purohit helped to draft the manuscript. All authors read and approved the final manuscript. We thank Veterinary Pathology Laboratory, Veterinary Clinical Complex and authority of Veterinary University, Mathura-281001, Uttar Pradesh, India for great support in completing the research study and all the animals and animal owners involved in the study.

References

1. Bray F, McCarron P, Parkin DM. The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res. 2004;6(6):229-39.
2. Gupta K, Sood NK, Uppal SK, Mohindroo J, Mahajan S, Raghunath M, et al. Epidemiological studies on canine mammary tumor and its relevance for breast cancer studies. IOSR J Pharm. 2012;2(2):322-33.
3. Varallo GR, Gelaleti GB, Signorini LBM, Moschetta MG, Lopes JR, De Nardi AB, et al. Prognostic phenotypic classification for canine mammary tumors. Oncol Lett. 2019;18(6):6545-53.

4. Gray M, Meehan J, Martínez-Pérez C, Kay C, Turnbull AK, Morrison LR, et al. Naturally-occurring canine mammary tumors as a translational model for human breast cancer. Front Oncol. 2020;10:617.
5. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, et al. Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res. 2010;12(4):207.
6. Thangathurai R. Cytological, histological, ultrastructural, molecular and immunohistochemical studies on canine mammary tumours. MVSc thesis submitted to Tamil Nadu Veterinary and Animal Sciences University, India; 2004.
7. Queiroga FL, Raposo T, Carvalho MI, Prada J, Pires I. Canine mammary tumours as a model to study human breast cancer: Most recent findings. *In Vivo*. 2011;25(3):455-65.
8. Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. Methods Mol biol. 2014;1180:31-43.
9. Luna LG. Manual of histologic staining methods of the Armed Forces Institute of Pathology; 1968.
10. Goldschmidt M, Peña L, Rasotto R, Zappulli V. Classification and grading of canine mammary tumors. Vet Pathol. 2011;48(1):117-31.
11. Peña L, Andrés PD, Clemente M, Cuesta P, Pérez-Alenza MD. Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. Vet Pathol. 2013;50(1):94-105.
12. Coindre JM. Immunohistochemistry in the diagnosis of soft tissue tumors. Histopathology. 2003;43(1):1-6.
13. Peña L, Gama A, Goldschmidt MH, Abadie J, Benazzi C, Castagnaro M, et al. Canine mammary tumors: A review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. Vet Pathol. 2014;51(1):127-45.
14. Mainenti M, Rasotto R, Carnier P, Zappulli V. Oestrogen and progesterone receptor expression in subtypes of canine mammary tumours in intact and ovariectomised dogs. Vet J. 2014;202(1):62-8.
15. Peña L, Dolores Perez-Alenza M, Rodriguez-Bertos A, Nieto A. Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases. Breast Cancer Res Treat. 2003;78(2):141-8.
16. Sorenmo KU, Kristiansen VM, Cofone MA, Shofer FS, Breen AM, Langeland M, et al. Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. Vet Comp Oncol. 2009;7(3):162-72.
17. Reddy GB, Kumar P, Kumar R, Pawaiya RV, Ravindran R. Histopathological classification and incidence of canine mammary tumours. Indian J Vet Pathol. 2009;33(2):152-5.
18. Srivastava AK, Singh B, Srivastava AK, Sinha N, Sharma AK. Canine neoplasms: A study on occurrence and distribution pattern. Indian J Vet Surg. 2009;30(1):45-46.
19. Sleeckx N, De Rooster H, EJ VK, Van Ginneken C, Van Brantegem L. Canine mammary tumours, an overview. Reprod Domest Anim. 2011;46(6):1112-31.
20. Moulton JE, Taylor DO, Dorn CR, Andersen AC. Canine mammary tumors. Pathol Vet. 1970;7(4):289-320.
21. Pastor N, Ezquerra LJ, Santella M, Caballé NC, Tarazona R, Durán ME. Prognostic significance of immunohistochemical markers and histological classification in malignant canine mammary tumours. Vet Comp Oncol. 2020;18(4):753-62.
22. Port Louis LR, Varshney KC, Nair MG. An immunohistochemical study on the expression of sex steroid receptors in canine mammary tumors. ISRN Vet Sci. 2012;2012.
23. Rutteman GR, Misdorp W, Blankenstein MA, Van den Brom WE. Oestrogen (ER) and progestin receptors (PR) in mammary tissue of the female dog: Different receptor profile in non-malignant and malignant states. Br J Cancer. 1988;58(5):594-9.
24. Brandão YD, Toledo MB, Chequin A, Cristo TG, Sousa RS, Ramos EA, et al. DNA methylation status of the estrogen receptor α gene in canine mammary tumors. Vet Pathol. 2018;55(4):510-16.
25. Ressel L, Puleio R, Loria GR, Vannozzi I, Millanta F, Caracappa S, et al. HER-2 expression in canine morphologically normal, hyperplastic and neoplastic mammary tissues and its correlation with the clinical outcome. Res Vet Sci. 2013;94(2):299-305.
26. Campos LC, Silva JO, Santos FS, Araújo MR, Lavalle GE, Ferreira E, et al. Prognostic significance of tissue and serum HER2 and MUC1 in canine mammary cancer. J Vet Diagn Invest. 2015;27(4):531-5.
27. Lee, Chung Ho, Wan Hee Kim, Ji Hey Lim, Min Soo Kang, Dae Yong Kim, and Oh Kyeong Kweon. "Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors." J Vet Sci. 2004;5(1):63-9.
28. Dileepkumar KM, Maiti SK, Naveen K, Ninu AR, Mitra A. Expression of p-53 and Cox-2 genes as tumour markers in spontaneous canine mammary tumours. Adv Anim Vet Sci. 2016;4:294-300.
29. Vural SA, Vural MR, Kuplulu S, Alcigir ME, Kaymaz M. Immunohistochemical demonstration of p53 protein and metallothioneins in canine mammary tumours. Revue Méd. Vét. 2009;160(6):300-7.
30. Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Król M, Jurka P. Current biomarkers of canine mammary tumors. Acta Vet Scand. 2018;60(1):1-3.
31. Kim NH, Lim HY, Im KS, Shin JI, Kim HW, Sur JH. Evaluation of clinicopathological characteristics and oestrogen receptor gene expression in oestrogen receptor-negative, progesterone receptor-positive canine mammary carcinomas. J Comp Pathol. 2014;151(1):42-50.