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Immunohistochemically Evaluation of PCNA and MMP-9 Expressions in Different Types of Canine Mammary Carcinomas

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Abstract

The aim of this study was to evaluate reveal whether there is a significant relationship between cell proliferation and invasion-metastasis capacity in various mammary carcinomas subtypes observed in dogs immunohistochemically with PCNA and MMP-9 markers. The material of this study consisted of tissue samples of mammary carcinomas taken from 6 Kangal and 4 Setter dogs with an average age of 8.4 years brought to our department between 2012 and 2020. 4 of 10 mammary carcinoma cases were classified as intraductal papillary carcinoma, 4 as carcinoma mixed, 1 as solid carcinoma and 1 as tubular carcinoma. We found the highest mean number of PCNA and MMP-9 positive cells in solid subtype. In conclusion, there may be a positive relationship between cell proliferation, invasion and metastasis capacity under the solid subtype.

Key Words: Carcinoma, dog, mammary, MMP-9, PCNA

Köpek Meme Karsinomlarında PCNA ve MMP-9 Ekspresyonlarının İmmunohistokimyasal Olarak Değerlendirilmesi

Öz

Bu çalışmanın amacı, köpeklerde görülen çeşitli meme karsinomu alt tiplerinde hücre proliferasyonu ile invazyon-metastaz kapasitesi arasında anlamlı bir ilişki olup olmadığını PCNA ve MMP-9 gibi immunohistokimyasal belirteçler aracılığıyla ortaya koymaktır. Bu çalışmanın materyali, 2012-2020 yılları arasında anabilim dalımıza getirilen yaş ortalaması 8,4 yıl olan 6 Kangal ve 4 Setter köpeğinden alınan meme kanseri doku örnekleri oluşturdu. 10 meme karsinomu vakasından 4'ü intraduktal papiller karsinom, 4'ü miks karsinom, 1'i solid karsinom ve 1'i tübüler karsinom olarak sınıflandırıldı. Solid alt tipte en yüksek ortalamada PCNA ve MMP-9 pozitif hücre sayısı bulundu. Sonuç olarak sadece solid alt tipte hücre proliferasyonu ile invazyon ve metastaz kapasitesi arasında pozitif bir ilişki olabileceği kanısına varıldı.

Anahtar Kelimeler: Karsinom, köpek, meme, MMP-9, PCNA

INTRODUCTION

The most common malignant tumors observed in bitches are mammary cancers and cause serious clinical problems (1-3). Although many factors play a role in the etiology of mammary tumors in dogs, it is thought that carcinogenic effect, several molecular pathways, e.g. dysregulation of cellular proliferation, lack of apoptosis and hormone dependence contribute significantly to neoplastic transformation (4-6). Surgery is the most-cost effective and primary treatment method (7). However, there is a low survival rate in bitches with malignant tumors. Because the recurrence rate of the tumor is very high and it can metastasize to organs such as regional lymph nodes, lung, liver and spleen (8, 9). Early diagnosis is the best strategy to fight mammary cancers (10). The most important prognostic factors related to the tumor can be briefly summarized as follows; tumor size, lymph node status, distant organ metastases, histological subtypes, degree of malignancy, and nucleus differentiation (11, 12). Mammary cancers have a wide variety of biological behaviors (13). Among these factors, histological subtypes are thought to be the best option for detecting the behavior of the tumor (14). Malign epithelial tumor types; carcinoma-in situ, carcinoma simple (tubular, tubulopapillary, cystic-papillary, cribriform), carcinoma-micropapillary invasive, carcinoma-

solid, comedocarcinoma, carcinoma-anaplastic, carcinoma-complex type, carcinoma and myoepithelioma, malignant myoepithelioma, carcinoma-mix type, ductal carcinoma and intraductal papillary carcinomas (15).

Proliferating cell nuclear antigen (PCNA) plays serious roles in the metabolism of nucleic acid. Its major function is in DNA replication, but it is also related with DNA excision repair, cell cycle control, chromatin assembly, and RNA transcription (16). Matrix metalloproteinases (MMPs) are zinc dependent proteolytic metalloenzyme. Matrix metalloproteinase-9 (MMP-9), also known as gelatinase b, is one of the most complex forms of matrix metalloproteinases. MMP-9 has the ability to break down the extracellular matrix (ECM) components and has serious role in the pathophysiological functions (17).

In this study, we aimed to evaluate whether there is a significant relationship between cell proliferation and invasion-metastasis capacity in various mammary carcinoma subtypes observed in dogs, by means of immunohistochemical markers such as PCNA and MMP-9.

MATERIALS AND METHODS

Animals

The material of this study consisted of tissue samples of mammary carcinomas taken from 6 Kangal and 4 Setter dogs with an average age of 8.4 years brought to our department between 2012-2020.

Ethical Approval

The ethics committee report of this study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (Authorization number: KAU-HADYEK-2020/076).

Histopathology

Tissue samples from dogs were fixed in 10 % buffered formalin solution. After routine tissue procedures, paraffin blocks were prepared and sections with a thickness of 5 µm were taken for Hematoxylin and Eosin (H&E) staining. Sections were examined with the light microscope to determine the histopathological subtypes and photographed with Cell^P Program.

Immunohistochemistry

Avidin-Biotin Peroxidase method was used as immunohistochemical method. For immunohistochemical staining, the sections of 4 µm in thickness taken to poly-L-lysine coated slides were deparaffinized and rehydrated in graded alcohols. In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide solution in Phosphate Buffered Saline (PBS) for 15 minutes. For antigen retrieval, the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the microwave oven (at 800 watt). In order to prevent nonspecific staining, the sections were incubated for 30 min with non-immune serum (Genemed Biotechnologies REF 54-0003) at room temperature. Diluted antibodies (PCNA: Santa Cruz, sc-56, Dilution Ratio: 1/100; MMP-9: Santa Cruz, sc-393859, Dilution Ratio: 1/100) were incubated for one hour at room temperature. The sections were washed 3 times in PBS solution for 5 minutes, and the biotinylated secondary antibody (Genemed Biotechnologies REF 54-0003) was applied to them at room temperature for 30 minutes. After washing in PBS (3-5 min), all sections were incubated with peroxidase-bound Streptavidin (Genemed Biotechnologies REF 54-0003) for 30 minutes at room temperature. A solution of 3.3-diaminobenzidine tetra hydrochloride (DAB) (Genemed Biotechnologies REF 10-0048) was used as a chromogen for 15 minutes. The sections were treated with Mayer's Hematoxylin for 30 second and washed in running water for 5 min, dehydrated in graded alcohols, cleared in xylene and coated with entellan. Primary antibodies were omitted from the negative control sections and were treated with diluted normal serum. The slides prepared after the covering were examined under a light microscope and photographed via the Cell^P program. Analyzes of the images were done with Image J Program.

Analysis of immunohistochemical staining results; PCNA and MMP-9 immunoreactivities were scored by number of positive cells in the areas that best reflect the character of staining. For quantification of the immunostaining in the tissue, the analysis was started on the basis of high intensity reaction areas. For each sample, 10 different areas were examined at a total enlargement of 200. The number of cells stained positively in each area was recorded and the average of these 10 sites was taken as the data of that animal.

RESULTS

Histopathological Results

4 of 10 mammary carcinoma cases were classified as intraductal papillary carcinoma, 4 as carcinoma mixed, 1 as solid carcinoma and 1 as tubular carcinoma according to Goldschmidt et al. 2017 (15). In histopathological examinations; we observed finger-like protrusions in which the tumor cells were supported by a fibrovascular layer in intraductal papillary carcinoma subtype cases. In addition, we detected changes in the ratio of nucleus and cytoplasm, increased mitotic activity and remarkable pleomorphism in neoplastic epithelial cells. In cases with carcinoma mixed subtype; we determined the presence of epithelial cells forming irregular ductular structures, spindle-shaped myoepithelial cells and bone-cartilage tissues. In addition to these, increased mitotic activity and pronounced pleomorphism were other important findings we observed. We found that 1 or 2 cell thick tubular structures, pleomorphism, marked hyperchromasia and increased mitotic activity in case of tubular carcinoma subtype. In cases with solid carcinoma subtype case; the presence of cells in the form of solid layers was noteworthy. In addition, we detected differences in nuclear and cytoplasm ratios and varying degrees of mitotic figures. (Figure 1 A-D).

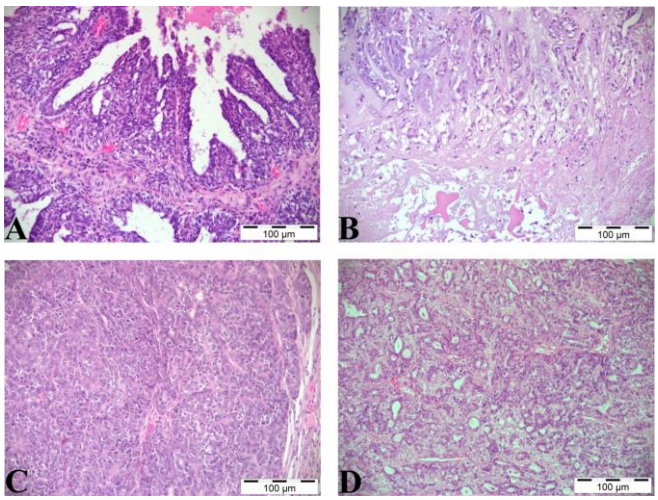

Figure 1. Histological subtypes, H&E, Bar=100 µm A) Intraductal papillary subtype. B) Carcinoma mix subtype. C) Solid subtype. D) Tubular subtype.

Immunohistochemical Results

Average numbers of positive PCNA, MMP-9 cells and metastasis status for all animals are given in Table 1. We found the highest mean number of PCNA positive cells in solid subtype. PCNA immunoreactivity was detected especially in the nucleus. In addition, cytoplasmic staining was observed. In cases with intraductal papillary carcinoma subtype; immune positive reactions were concentrated in the tumoral cells that formed finger-like protrusions compared to the fibrovascular layer. In cases with carcinoma mixed subtype; strong immunoreactivity was observed in epithelial cells forming irregular glandular structures. We did not detect any positive reaction in cartilage and bone tissue components. In case with tubular carcinoma subtype; PCNA expression especially was evident in the solid layers formed by the tumoral cells. In case with solid carcinoma subtype; immunpositive reaction was detected in pleomorphic tumoral cells forming tubular structures separated by thin fibrous tissue (Figure 2 A-D).

Table 1. Average PCNA and MMP-9 positive cell numbers of the cases

Case No	Histologic Subtype	PCNA	MMP-9	Metastasis
Case 1	Carcinoma mix type	421	392	-
Case 2	Carcinoma intraductal papillary type	781	462	-
Case 3	Carcinoma intraductal papillary type	839	492	+
Case 4	Carcinoma solid type	1273	623	+
Case 5	Carcinoma tubular type	1080	576	-
Case 6	Carcinoma mix type	486	372	-
Case 7	Carcinoma mix type	468	388	-
Case 8	Carcinoma intraductal papillary type	971	480	+
Case 9	Carcinoma intraductal papillary type	611	392	-
Case 10	Carcinoma mix type	517	406	-

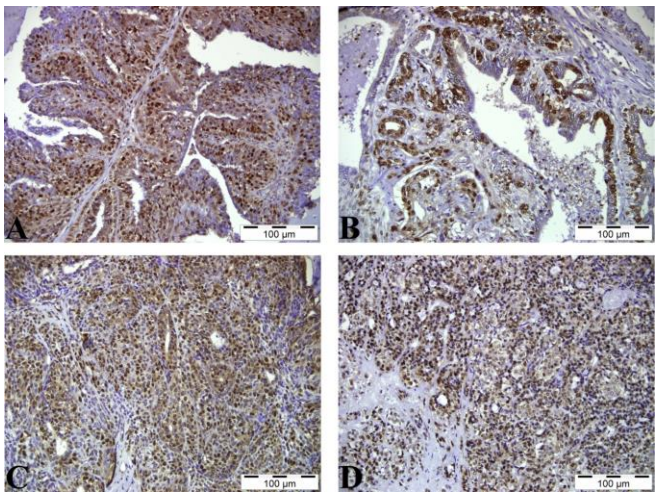

Figure 2. PCNA positive expressions, IHC, Bar=100 µm. **A)** Intraductal papillary subtype. **B)** Carcinoma mix subtype. **C)** Tubular subtype. **D)** Solid subtype

Similar to PCNA, we found the highest mean number of MMP-9 positive cells in solid subtype. MMP-9 immunoreactivity was observed in both the cytoplasm and the nucleus of tumoral cells. Positive reactions in the cases with intraductular papillary subtype were detected especially in the cells forming the glandular structures. In the cases with carcinoma mixed subtype, no reaction was observed in bone and cartilage tissue components similar to PCNA, while brown positive reactions were detected especially in the tumoral cells between these two components. A positive reaction was detected in a case with tubular subtype spread over large areas. In the case with solid subtype, MMP-9 expressions were observed densely in the cytoplasm of cells forming solid sheets separated by a thin fibrous tissue (Figure 3 A-D).

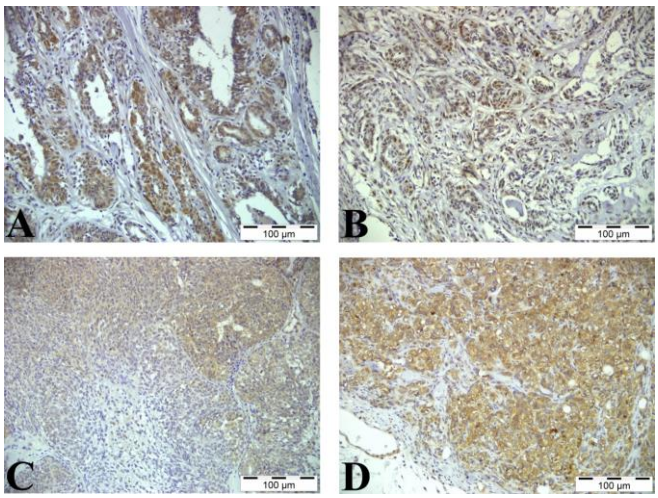

Figure 3. MMP-9 positive expressions IHC, Bar=100 µm. **A)** Intraductal papillary subtype. **B)** Carcinoma mix subtype. **C)** Tubular subtype. **D)** Solid subtype.

DISCUSSION AND CONCLUSION

Uncontrolled cell proliferation is one of the most important reasons for the transformation of normal cells into malignant cells (18). Proliferation index can be determined by cell

cycle markers such as PCNA and Ki-67. Compared to Ki-67, PCNA is a more reliable marker for mammary tumors in humans and dogs (1). This is due to the long half-life of PCNA in cells. It is thought to take approximately 8 hours for dividing cells and approximately 20 hours for cells that enter the resting phase (18, 19). PCNA is an acid nuclear protein weighing 36 kDa and working as DNA polymerase delta co-factor. In addition, it plays an important role in DNA replication and repair processes (20). PCNA is synthesized in the G1/S phases of cell division, and its expression is closely associated with cell proliferation (21, 22). Subsequently PCNA decreases in the G2/M phases (19, 23). Increased PCNA expression is associated with mitotic activity, poor prognosis and malignant potential; PCNA positive reaction detected at a rate of 50% or more in malignant cells indicates poor prognosis (4, 24). While studies highlight the prognostic value of cell proliferation in canine mammary tumors, some of the results obtained are open to discussion (13).

Preziosi et al. 1995 (19), Peña et al. 1998 (22), Zacchetti et al. 2003 (23) and Silva et al. 2016 (25) demonstrated PCNA immunoreactivity both intensely and weakly in the nucleus, they also found a positive reaction in the cytoplasm of cells that undergoing mitosis. In addition, Silva et al. 2016 (25) found that PCNA immunostaining reacted more intensely in the nuclei of tumoral cells in the mammary glands compared to connective tissue and myoepithelial cells. They did not find any PCNA expression in bone and cartilage-derived myxoid areas (25). In accordance with this study data, we did not detect any positive reaction in cartilage and bone tissue components in cases with carcinoma mixed subtype. Similar to previous studies, İlhan et al. 2008 (24) and Carvalho et al. 2016 (13) observed PCNA immunoreactivity especially in the nucleus of cells. Zuccari et al. 2008 (20) observed intense and nuclear PCNA expressions in canine mammary neoplasies. Aydogan et al. 2018 (1) detected the PCNA positive reaction in the nucleus and brown color. In this study, in accordance with the literature data, we observed PCNA positive reactions in the brown color in the nucleus of tumor cells (13, 24). In addition, similar to previous studies, we detected PCNA expressions in the cytoplasm of cells undergoing mitosis (19, 22, 23, 25). Similar to the study conducted by Ilhan et al. 2008 (24), we did not detect any PCNA positive reaction in normal mammary glands.

Preziosi et al. 1995 (19) found that PCNA positive cells are higher in solid adenocarcinomas than tubular adenocarcinomas. Funakoshi et al. 2000 (21) observed the highest PCNA expression in solid type, while the lowest reaction was found in tubular type. İlhan et al. 2008 (24) found the intensity of PCNA staining higher in papillary adenocarcinomas compared to solid adenocarcinomas. Ranganath et al. 2011 (9) detected the highest PCNA expressions in solid carcinomas. Solid carcinomas were followed by papillary, tubular and complex carcinomas, respectively. Lokesh et al. 2014 determined that the PCNA index was higher in solid carcinoma cases compared to tubulopapillary and complex adenocarcinomas (26). Łopuszyński and Hellmén 2015 (18) and Carvalho et al. 2016 (13) detected the highest mean PCNA immunoreactivity in solid carcinomas, followed by tubulopapillary carcinomas and complex carcinomas, respectively. Aydoğan et al. 2018 (1) observed PCNA positive reactions in the following histological subtypes of carcinomasarcomas, tubulopapillary carcinoma and complex carcinomas, respectively. Similar to previous studies, we found the highest mean number of PCNA positive cells in tubular subtype (9, 13, 18, 19, 21, 26).

In canine tumors, invasion to surrounding tissues and metastasis to other organs are quite common (27). Invasion and metastasis occur as a result of a highly complex and multi-stage process (28). The first of these stages is the destruction of the extracellular matrix and the invasion of the basement membrane (28, 29). Various proteolytic systems mediate the destruction of these elements and especially MMPs play a significant role (30). MMPs are a zinc-dependent family of proteases and are synthesized mainly by fibroblasts, leukocytes, monocytes, macrophages, neutrophils and endothelial cells as well as neoplastic cells and released into extracellular spaces (2, 29). The MMP family consists of several substrate-specific subfamilies; gelatinases, one of them, is a subfamily often associated with tumor invasion and metastasis. Gelatinases break down the type 4 collagen of the basement membrane and thus contribute to invasion, tumor growth and spread (30). MMP-9 (also known as gelatinase b), which is one of the most important members of gelatinases and weighs 92-kDa, is involved in cancer initiation, development, metastasis, and progression (10). The increase in MMP-9 activity observed in canine mammary tumors is closely related to tumor malignancy, proliferation rate and histological grade (30).

Chen et al. 2019 (10) found that malignant canine breast tumors express MMP-9 at a higher rate than normal breast tissues. Nowak et al. 2008 (29) detected MMP-9 positive reaction in 83% of the cases in their study with tissue samples taken from 35 canine mammary carcinomas. Santos et al. 2012 (30) found MMP-9 expressions in both stromal and neoplastic cells in 31 solid and 20 tubulopapillary canine adenocarcinoma cases. Hirayama et al. 2002 (27) observed MMP-9 expressions particularly strongly in the cytoplasm of neoplastic luminal cells in tubular, papillary and solid adenocarcinoma cases. Similar to this study Aresu et al. 2011 (28) MMP-9 positive reactions were detected in the cytoplasm of tumoral cells and fibroblasts. Raposo et al. 2016 (31) reported that in canine inflammatory mammary carcinomas, MMP-9 expressions are localized in the cytoplasm of neoplastic cells. Dong et al. 2019 (2) detected 85.3% of MMP-9 positivity in 34 tubulopapillary canine breast cancer cases. In accordance with literature data, we detected MMP-9 positive reaction in all cases of intraductal papillary, mixed, tubular and solid subtypes (27-30). Similar to previous studies (27, 28), we found MMP-9 expressions especially in the cytoplasm of neoplastic cells. Apart from cytoplasms, we also observed a positive reaction in the nuclei of some cells. In the literature searches, there is no study comparing different canine mammary carcinoma subtypes in terms of MMP-9 expressions. In our study, the subtype with the highest mean number of MMP-9 positive cells was solid type parallel to the number of PCNA positive cells. When the metastasis status

of animals is examined, it is seen that only 3 out of 10 cases metastasize to near or distant tissues. Of the 3 cases that metastasize, 2 are intraductal papillary and 1 case is solid type.

In conclusion, there may be a positive relationship between cell division rate, invasion and metastasis capacity under the solid subtype. Although the tubular type PCNA and MMP-9 positive cell count scored higher than the intraductal papillary variant, we did not detect any metastasis in this case. We interpreted this result in relation to the small number of samples studied. We think that the correlation of PCNA and MMP-9 expressions with each other may be very valuable, especially in terms of tumor aggressiveness, cancer prognosis and histologic subtypes.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest with respect to the publication of this manuscript.

REFERENCES

1. Aydogan A, Ozmen O, Haligur M, Sipahi C, Ileri D, Haligur A. (2018). Immunohistochemical Evaluation of Bcl-2, ER-alpha, Caspase-3,-8,-9, PCNA and Ki-67 Expressions in Canine Mammary Carcinomas. Biotech Histochem. 93(4): 286-292.
2. Dong H, Diao H, Zhao Y, et al. (2019). Overexpression of Matrix Metalloproteinase-9 in Breast Cancer Cell Lines Remarkably Increases the Cell Malignancy Largely via Activation of Transforming Growth Factor Beta/SMAD Signalling. Cell Prolif. 52(5): e12633.
3. Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Król M, Jurka P. (2018). Current Biomarkers of Canine Mammary Tumors. Acta Vet Scand. 60(1): 60-66.
4. Vinothini G, Balachandran C, Nagini S. (2009). Evaluation of Molecular Markers in Canine Mammary Tumors: Correlation with Histological Grading. Oncol Res. 18(5-6): 193-201.
5. Dolka I, Król M, Sapierzyński R. (2016). Evaluation of Apoptosis-Associated Protein (Bcl-2, Bax, cleaved caspase-3 and p53) Expression in Canine Mammary Tumors: An Immunohistochemical and Prognostic Study. Res Vet Sci. 105: 124-133.
6. Kumaraguruparan R, Prathiba D, Nagini S. (2006). Of Humans and Canines: Immunohistochemical Analysis of PCNA, Bcl-2, p53, Cytokeratin and ER in Mammary Tumours. Res Vet Sci. 81(2): 218-224.
7. Muhammadnejad A, Keyhani E, Mortazavi P, Behjati F, Haghdoost IS. (2012). Overexpression of Her-2/Neu in Malignant Mammary Tumors; Translation of Clinicopathological Features from Dog to Human. Asian Pac J Cancer Prev. 13(12): 6415-6421.
8. Senhorello ILS, Terra EM, Sueiro FAR, et al. (2020). Clinical Value of Carcinoembryonic Antigen in Mammary Neoplasms of Bitches. Vet Comp Oncol. 18 (7069): 315–323.
9. Ranganath GJ, Kumar Ram, Vishwanatha Reddy AP, Mayilkumar K, Pawaiya RVS, Maiti SK. (2011). Comparative Study on the Expression Pattern of the Proliferating Cell Markers PCNA and Ki67 in Canine Mammary Tumours. Ind J Vet Pathol. 35(1): 13-17.
10. Chen YC, Chang SC, Huang YH, et al. (2019). Expression and the Molecular Forms of Neutrophil Gelatinase-associated Lipocalin and Matrix Metalloproteinase 9 in Canine Mammary Tumours. Vet Comp Oncol. 17(3): 427-438.
11. Veena P, Suresh Kumar RV, Raghavender KBP, Srilatha Ch, Rao TSC. (2014). Immunohistochemical Detection of p53 in Canine Mammary Tumors. Indian J Anim Res. 48(2): 204-206.
12. Klopfleisch R, Klose P, Weise C, et al. (2010). Proteome of Metastatic Canine Mammary Carcinomas: Similarities to and Differences from Human Breast Cancer. J Proteome Res. 9(12): 6380-6391.
13. Carvalho MI, Pires I, Prada J, Lobo L, Queiroga FL. (2016). Ki-67 and PCNA Expression in Canine Mammary Tumors and Adjacent Nonneoplastic Mammary Glands: Prognostic Impact by a Multivariate Survival Analysis. Vet Pathol. 53(6): 1138-1146.
14. Munday JS, Ariyarathna H, Aberdein D, Thomson NA. (2019). Immunostaining for p53 and p16CDKN2A Protein is not Predictive of Prognosis for Dogs with Malignant Mammary Gland Neoplasms. Vet Sci. 6(1): 34.
15. Goldschmidt MH, Peña L, Zappulli V. (2017). Tumors of the Genital Systems. In : Tumors of Domestic Animals. Moulton JE (ed), 5th ed. pp. 723-764, Wiley Blackwell, Iowa, USA.
16. Juríková M, Danihel L, Polák Š, Varga I. (2016). Ki67, PCNA, and MCM Proteins: Markers of Proliferation in the Diagnosis of Breast Cancer. Acta Histochem. 118(5): 544-552.
17. Mondal S, Adhikari N, Banerjee S, Abdul Amin SK, Jha T. (2020). Matrix Metalloproteinase-9 (MMP-9) and its Inhibitors in Cancer: A Minireview. Eur J Med Chem. 194: 112260.
18. Łopuszyński W, Hellmén E. (2015). Cell Proliferation Study in Canine Mammary Carcinomas. Int J Vet Health Sci Res. 3(2): 39-45.
19. Preziosi R, Sarli G, Benazzi C, Marcato PS. (1995). Detection of Proliferating Cell Nuclear Antigen (PCNA) in Canine and Feline Mammary Tumours. J Comp Path. 113(4): 301-313.
20. Zuccari DAPC, Pavam MV, Terzian ACB, Pereira RS, Ruiz CM, Andrade JC. (2008). Immunohistochemical Evaluation of E-cadherin, Ki-67 and PCNA in Canine Mammary Neoplasias: Correlation of Prognostic Factors and Clinical Outcome. Pesq Vet Bras. 28(4): 207-215.
21. Funakoshi Y, Nakayama H, Uetsuka K, Nishimura R, Sasaki N, Doi K. (2000). Cellular Proliferative and Telomerase Activity in Canine Mammary Gland Tumors. Vet Pathol. 37(2): 177-183.

22. Peña LL, Nieto AI, Pérez-Alenza D, Cuesta P, Castaño M. (1998). Immunohistochemical Detection of Ki-67 and PCNA in Canine Mammary Tumors: Relationship to Clinical and Pathologic Variables. J Vet Diagn Invest. 10(3): 237-246.
23. Zacchetti A, van Garderen E, Teske E, Nederbragt H, Dierendonck JH, Rutteman GR. (2003). Validation of the Use of Proliferation Markers in Canine Neoplastic and Non-Neoplastic Tissues: Comparison of KI-67 and Proliferating Cell Nuclear Antigen (PCNA) Expression Versus in Vivo Bromodeoxyuridine Labelling by Immunohistochemistry. APMIS. 111(3): 430-438.
24. İlhan F, Metin N, Türkütanıt Birincioğlu S. (2008). Immunohistochemical Detection of PCNA and p53 in Mammary Tumours and Normal Tissues in Dogs. Revue Méd Vét. 159(5): 298-304.
25. Silva FB, Leite Jda S, de Mello MF, Ferreira AM (2016). Validation of a Low-cost Modified Technique for Constructing Tissue Microarrays For Canine Mammary Tumor Analysis. Pathol Res Pract. 212(9): 783-790.
26. Lokesh JV, Kurade NP, Shivakumar MU, Sharma AK, Maiti SK. (2014). Evaluation of BcL-2 and PCNA Expression and Mitotic Index in Spontaneous Canine Tumors. Adv Anim Vet Sci. 2(1): 63-66.
27. Hirayama K, Yokota H, Onai R, et al. (2002). Detection of Matrix Metalloproteinases in Canine Mammary Tumours: Analysis by Immunohistochemistry and Zymography. J Comp Pathol. 127(4): 249-256.
28. Aresu L, Giantin M, Morello E, et al. (2011). Matrix Metalloproteinases and Their Inhibitors in Canine Mammary Tumors. BMC Vet Res. 7: 33.
29. Nowak M, Madej JA, Podhorska-Okolow M, Dziegiel P. (2008). Expression of Extracellular Matrix Metalloproteinase (MMP-9), E-cadherin and Proliferation-associated Antigen Ki-67 and Their Reciprocal Correlation in Canine Mammary Adenocarcinomas. In Vivo. 22(4): 463-469.
30. Santos AA, Lopes CC, Marques RM, Amorim IF, Gärtner MF, de Matos AJ. (2012). Matrix Metalloproteinase-9 Expression in Mammary Gland Tumors in Dogs and its Relationship with Prognostic Factors and Patient Outcome. Am J Vet Res. 73(5): 689-697.
31. Raposo TP, Beirão BC, Pires I, et al. (2016). Immunohistochemical Expression of CCR2, CSF1R and MMP9 in Canine Inflammatory Mammary Carcinomas. Anticancer Res. 36(4): 1805-1813.

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