RESEARCH NOTE

THE PRESENCE OF CSCs AND EMT MARKERS ARE POSITIVELY CORRELATED WITH CANINE MALIGNANT MAMMARY GLAND TUMORS IN IRAN

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ABSTRACT

Canine malignant mammary gland tumors (MMGTs) are one of the most prevalent neoplasia in bitches. Thus, this present work was designed to evaluate the correlation of cancer stem cells (CSCs) existence and epithelial-mesenchymal transition (EMT) phenomena with well-known prognostic factors, including Ki-67, TNM staging that determines tumor size (T), lymph nodes involvement (N) and distant metastases (M) as a whole factor, and tumor infiltrating lymphocytes (TILs) in 25 formalin-fixed paraffin embedded blocks (FFPEBs) of canine MMGTs. FFPEBs were cut, underwent immunohistochemistry staining with primary antibodies including Ki-67, vimentin (as an EMT representative), CD44, CD24, and ALDH1 for detecting CSCs. The Pearson Chi-Square and *t* tests were applied for finding statistical correlations among variables. Significant correlation was found between presence of CSCs and Ki-67 (P=0.004). Furthermore, a statistically significant correlation was shown between $CSCs^{+}/EMT^{+}$ cases with Ki-67 (P=0.006). Among three CSC markers, ALDH1 illustrated a positive correlation with EMT (P=0.015). No significant differences were found between presence of TILs and all other investigated variables such as CSCs, EMT, Ki-67, and TNM stages. Thus, for the first time in Iran, results showed the importance of the association between CSCs, EMT, and Ki-67 in relation to the emergence of malignant behavior of canine MMGTs.

Keywords: *Canine mammary gland tumors, Cancer stem cells, Ki-67, Epithelial-Mesenchymal transition*

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INTRODUCTION

The research interest in understanding the heterogeneous cellular compartments of mammary gland tumors and their correlations with clinicopathologic features have been increased in both veterinary and medical oncology. Canine MMGTs have been diagnosed in more than 50% of all mammary tumors in bitches and share multiple analogies with HBCs, including the similar criteria used for early diagnosis, treatment prediction, and prognosis (Philibert *et al.*, 2003; Ahmadi *et al.*, 2010; Aleskandarany *et al.*, 2015; Ayob and Ramasamy 2018; Kaszak *et al.*, 2018; Andonegui-Elguera *et al.*, 2020). Additionally, the spontaneous nature of canine MMGTs makes them favorable models

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to study the complexity of this cancer and even examine new therapeutic approaches proposed in medical oncology (Kaszak *et al.*, 2018). Thus, comparative oncology cannot only create a great opportunity to enhance the understanding of breast cancer complexities by bidirectional exchanging of the latest discoveries between veterinary and medical oncology, but can also lead to improving diagnostic and prognostic assessments in veterinary medicine (Queiroga *et al.*, 2011; Carvalho *et al.*, 2016; Rybicka and Król 2016; Nguyen *et al.*, 2018).

Since metastases-related deaths are still the major challenge in the management of cancer patients, there has been a growing body of literature on finding the potential origins of dormancy and subsequent distant metastases even after decades (Ahmadi *et al.*, 2010; Aleskandarany *et al.*, 2015). The heterogeneous mammary mass consists of different cellular populations with distinct effects on tumor behavior, among which a small cellular fraction called cancer stem cells (CSCs) have been recently attracted more attention as a main contributor in distant metastases and tumor relapses. Self-producing capacity, multi-lineage differentiation potential, and resistance to common therapeutic modalities are among the most significant features of this population (Wei *et al.*, 2016; Rogez *et al.*, 2019).

Cancer stem cells are divided into two groups based on their phenotypes and strength of spreading, and migratory and stationary CSCs. Migratory CSCs use different strategies of spreading throughout the body to form distant metastases. One of these strategies could be EMT which leads to more aggressive behavior including migration and resistance to anoikis and therapies (Apostolou *et al.*, 2012; Chaffer *et al.*, 2016).

Another significant determinant of the outcome of different malignancies, particularly canine MMGTs, is the proliferation index (PI) measured by the expression of a nuclear protein called Ki-67 (Caziuc *et al.*, 2019). Ki-67 is well-known for its prominent role in tumor cell proliferation and enhancing the tumor size. However, the functional importance of Ki-67 in maintaining the CSCs population and affecting the tumor aggressiveness in tandem with CSCs needs to be elucidated (Cidado *et al.*, 2016).

The other effective cellular compartment in tumor is tumor infiltrating lymphocytes (TILs) with prognostic and predictive values. TILs may exert their impacts on tumor behavior by forming functional networks with other cells, particularly CSCs (Wei *et al.*, 2016). They are well studied in HBCs (Caziuc *et al.*, 2019) however, their significance and correlation with CSCs in canine MMGTs have not been fully understood.

Although many researches have been conducted in this field throughout the world, it is noteworthy that the present study was the first research in Iran which focuses on discovering the correlations among CSCs and canine MMGTs' clinicopathologic features, particularly Ki-67, and evaluating the correlation between tumor size and CSC^{+}/EMT^{+} cells by using immunohistochemical staining.

MATERIALS AND METHODS

Histopathological assessment of canine MMGTs

This cross-sectional study utilized canine MMGTs archived in University of Tehran, Faculty of Veterinary Medicine, Tehran, Iran, of which 25 cases of MMGCs and their paraffin-embedded blocks were chosen. TNM stagings were done based on Cassali *et al.* (2011) for 25 cases. One block per patient was selected for TIL assessment. Blocks were cut in 4 µm then, the prepared slides were stained with hematoxylin-eosin (H&E). H & E stained slides were evaluated under 200-400 X magnification. All mononuclear immune cells specifically in stromal parts of tumor are defined and scored as stromal TILs (Figs. 1-2). Stromal TILs were reported as percentages, including 0-10%, 10-40%, 40-60%, and more than 60%. The whole tumor mass surrounded by invasive margins should be considered in TILs evaluation (Caziuc *et al.*, 2019).

Immunohistochemical assessment of CSCs, EMT, and Ki-67

The well-accepted CSC phenotype in mammary gland mass is $ALDH1^{+}/CD44^{+}/CD24^{Low/-}$ in both canine and human (Michishita *et al.*, 2012). The routine method for detection of this population is using immunohistochemical staining on 3-4 µm sections of tissue samples. Thus, all tissue slides were first de-paraffinized in xylene and rehydrated in graded alcohol. Then, these were immersed in hydrogen peroxide in order to block endogenous peroxidase. Thereafter, slides were placed in microwave for antigen retrieval and incubated with primary antibodies, including ALDH1 (dilution; 1:50 - 1:500, Santa Cruz, USA), CD44 (dilution; 1:200 - 1:1000, Novus, USA), CD24 (dilution; 1:50 - 1:500, Santa Cruz, USA) for detecting CSCs, Ki-67 (Biocare Medical, California, USA) as a proliferation index and Vimentin (dilution; 1:100 - 1:1000, Santa Cruz, USA) for EMT assessment, for almost one hour at room temperature. The next step was exposure to

secondary antibody and HRP detection kit (Biocare Medical, California, USA) which was followed by antigen-antibody reactions amplification by using Betazoid DAB. Then, the sections were counterstained with hematoxylin and rinsed in tap water, dehydrated by alcohol, and cleared by xylene. Immunohistochemical evaluation was done based on modified Alred Scoring System as a semi- quantitative analysis of carcinoma cells with cut-off point ≥ 1%. ALDH1 as a cytoplasmic antigen and CD44 and CD24 as membranous antigens were estimated with respect to mentioned cut-point. Positive and negative controls were done based on companies' protocols.

Proliferation index was analyzed according to the presence of nuclear immunostaining in 1,000 epithelial cancer cells. PI was graded as following categories: low (< 5%), moderate (5 - 15%), and high (> 15%) (Goldhirsch *et al.*, 2011).

Vimentin expression was assessed in carcinoma cells based on presence and intensity of immunoreaction in the cytoplasm of 1,000 cells in 10 HPFs. Internal negative controls were endothelial cells and fibroblasts.

Statistical analyses:

SPSS software (v. 24; Armonk, NY: IBM Corp (2016), USA) was used for determining the correlations between CSCs and Ki-67, CSC/EMT presence and Ki-67, tumor size and CSC/EMT expression. The Pearson Chi-Square test was used to analyze the correlations between qualitative parameters such as CSCs, EMT, TILs, and tumor size. The *t* test was used to find the correlations between quantitative parameters such as Ki-67 and CSCs/EMT. The results of the present study were presented as mean ± standard deviation (SD). It should be noted that P-value < 0.05 was considered significant.

RESULTS

According to the TNM staging system, the majority of cases in the present study were Stage I (52%), and the percentages of Stages II, III and IV were as follows; 20%, 12%, and 16%, respectively. Given to accepted CSC phenotype, ALDH1 expression was shown in the cytoplasm, and CD44 and CD24 immunostaining were mainly detected at cell membranes of epithelial cancer cells (Figs. 3-5). Further analyses illustrated that only one of the CSC positive cases was in Stage I, while other six CSC positive cases belonged to Stage II (two cases), III (two cases), and IV (two cases). Among three parameters forming TNM stage, only the tumor size showed statistically significant association with CSC and EMT markers expression (P= 0.018).

Ki-67 labeling was exclusively detected in the nuclei of mammary cancer cells (Fig. 6) and was significantly lower in Stage I tumors compared to Stage II (P=0.025), III (P=0.018), and IV (P=0.011). Table 1 shows the summary of all data gained in this work.

The most striking result in the present study was a significant positive correlation between Ki-67 expression and the presence of CSCs (P=0.004). Interestingly, for those cases possessing CSCs, average percentages of Ki-67 were higher compared to ones devoid of CSCs.

Analysis of vimentin expression, as a representative of EMT, indicated 68% positivity (Fig. 7). To explore potential strength of concurrent presence of EMT and CSCs, further analyses showed statistically significant correlation between CSC^+/EMT^+ and canine MMGC proliferation rate (Ki-67) compared to $CSCs^-/EMT^-$ cases (P=0.006). Additionally, these $CSCs^+/EMT^+$ cases demonstrated a positive correlation with tumor size (P=0.018).

Analyzing the correlation of different CSCs markers individually with EMT demonstrated that only ALDH1 had a strong positive correlation with vimentin expression (P= 0.015).

TILs evaluation showed 72% of cases ≤10% TILs, 24% of cases 10%< TILs ≤ 40%, 4% of cases 40% < TILs ≤ 60%, and 0% TILs ≥ 60%. Among seven CSC^+ cases, just one case had 10-40% TILs and the rest had 0-10% TILs.

DISCUSSION

Increasing body of evidence proposed CSCs as the root of challenges facing the management of cancer patients and most of therapy failures (Cui *et al.*, 2015). Although different works have been carried out in cancer stem cell biology, many aspects of their interactions with other elements present in a tumor still remain unclear. Thus, designing further researches in order to gain deep comprehension of these problematic cellular networks are warranted and could open the way of implementing more precise target therapies.

In the present work, we primarily focused on investigating the correlation between the presence of CSCs and Ki-67 in canine MMGTs, in which immunohistochemical staining showed significant positive association. This finding is in agreement with recent studies. For instance, Cidado *et al.* (2016) reported the decrease in CSCs'

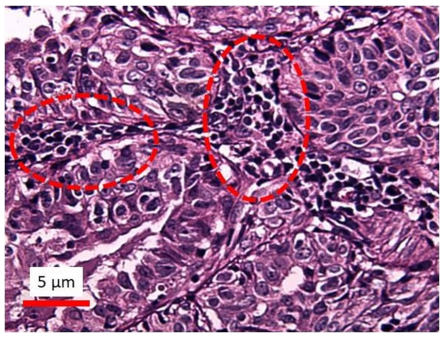

Figure 1. Tumor infiltrating lymphocytes in adjacent mammary gland cancer cells are shown in red dotted circles. Canine MMGTs are illustrated in glandular structures (H&E. ×400)

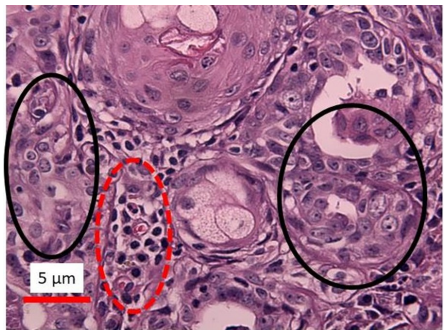

Figure 2. Neoplastic mammary gland cells are present in the black solid circles; Tumor infiltrating lymphocytes is shown in the stromal part of canine MMGT in the red dotted circle (H&E)

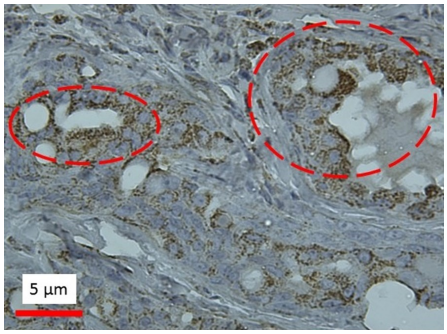

Figure 3. The high immunoexpression of ALDH1 is illustrated in red dotted circles in invasive canine MMGT cells; the brown pigmentation in the cytoplasm of epithelial cancer cells is suggestive of ALDH1 expression (IHC)

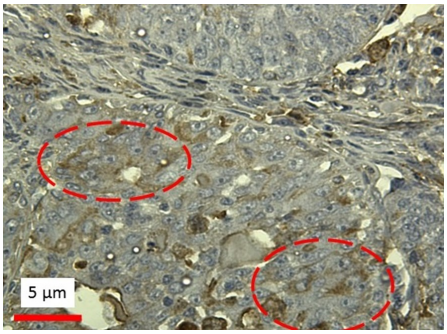

Figure 4. The moderate immunoexpression of CD44 is shown in red dotted circles in invasive canine MMGT cells; epithelial cancer cells with brown pigmentation in their cell membranes are indicative of CD44 expression (IHC)

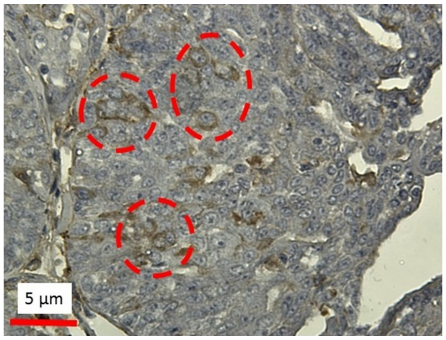

Figure 5. The immunoexpression of CD24 is shown in red dotted circle in canine MMGT cells; epithelial cancer cells with brown pigmentation in their cell membranes are suggestive of CD24 expression (IHC)

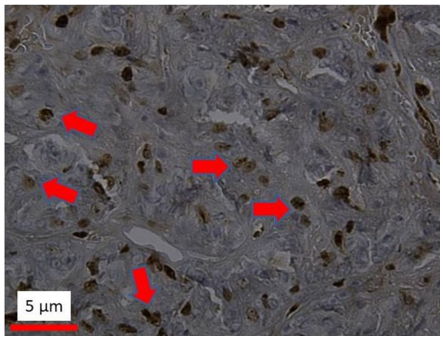

Figure 6. High Ki-67 immunostaining as a dense brown nuclear pigmentation of epithelial cancer cells in canine MMGT are shown by red solid arrows (IHC)

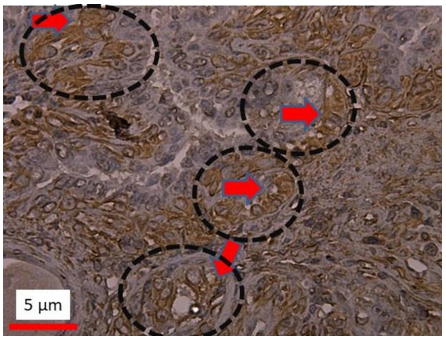

Figure 7. High vimentin immunoexpression in the cytoplasm of epithelial cancer cells in canine MMGT are illustrated by red solid arrows; the glandular structure of MMGT in some parts of figure are shown by black dotted circles which can confirm the epithelial origin of vimentin expression rather than mesenchymal stromal cells (IHC)

Table 1. Clinicopathologic and immunohistochemical data of canine MMGT samples (n= 25).

Case number	TNM Staging	TIL	Ki-67	Tumor Size (cm)	Vimentin	Cancer Stem Cell Markers			
						ALDH1	CD44	CD24	CSC status
1	Stage I	I	4-6%	1.7	P	1	0	0	-
2	Stage I	I	0.13	2.8	P	1	0	0	-
3	Stage IV	II	8-10%	2.4	N	1	0	0	-
4	Stage II	I	0.09	4.2	P	1	1	0	+
5	Stage I	I	0.06	1.7	N	1	0	0	-
6	Stage I	I	0.11	2.5	P	1	0	0	-
7	Stage IV	I	38-40%	8	P	1	1	0	+
8	Stage II	I	0.05	3.3	N	1	0	0	-
9	Stage I	III	0.1	2.5	P	1	0	0	-
10	Stage IV	I	0.37	4.6	P	1	1	0	+
11	Stage IV	II	19-21%	1.6	N	1	0	0	-
12	Stage II	I	0.12	3.8	P	1	0	1	-
13	Stage II	I	0.12	4	P	1	1	0	+
14	Stage I	I	0.09	1.6	P	1	0	0	-
15	Stage I	I	14-16%	2.2	N	1	0	0	-
16	Stage I	II	0.05	1.8	P	1	0	0	-
17	Stage I	II	0.08	1.9	P	1	0	1	-
18	Stage III	I	0.24	6.3	P	1	0	0	-
19	Stage I	II	0.1	2.2	P	1	0	0	-
20	Stage III	I	0.16	5.2	P	1	1	0	+
21	Stage I	I	0.04	1	N	1	0	0	-
22	Stage I	II	0.18	2	N	1	1	0	+
23	Stage III	I	0.21	2	P	1	1	0	+
24	Stage I	I	0.29	1.8	P	1	0	0	-
25	Stage II	I	0.04	3.3	N	0	0	0	-

markers in human malignancies because of genetic manipulation and knock down of Ki-67. Additionally, Saricanbaz *et al.* (2014) found the collaborative effects of both CSCs and Ki-67 on creating larger tumor dimensions, advanced clinical TNM stages, and multiple metastases in cancer patients**.** Furthermore, based on Cui *et al.* (2015), the strong positive correlation among $ALDH1^{High+}/CD44^{+}$ CSCs and high expression rate of Ki-67 in HBC was shown. This type of association could be attributed to the proliferative ability of CSCs based on their unique self- renewal capacity and transdifferentiation to different cell types in a tumor bulk. Another possible explanation according to Mrouja *et al.* (2021) findings is that Ki-67 plays multiple roles in carcinogenesis, tumor progression, and finally metastatic colonization, which all are in agreement with CSCs features. These pivotal roles of Ki-67 protein include empowering cancer cells in adjusting to their surroundings, participating in cancer cell interactions with immune system, creating the hub-like networks with multiple transcription factors involving in metastatic cascades. However, Paula *et al.* (2016) demonstrated a converse relation between $ALDH1^{+}/CD44^{+}$ CSCs and Ki-67 expression in patients with distant metastases. According to Floor *et al.* (2011) findings, CSCs are constantly changing from quiescent status to proliferative form by rearrangement of cell programs based on situations in which they want to survive. This cellular plasticity can be affected by different factors, including genetic and chromosomal configuration as the intrinsic contributors, and presence of growth factors, sufficient oxygen and nutrients, and blood vessels as the extrinsic effectors. All of these elements can exert significant effects on the fate and behavior of CSCs.

The study showed significant correlation between tumor size and CSC/EMT positive cases, which is completely justifiable with respect to higher proliferation rate and subsequent growing tumor dimensions in these cases (Apostolou *et al.*, 2012; Mansour and Atwa 2015; Lathia *et al.*, 2020). This positive correlation can propose the CSC/EMT status of canine MMGTs as a significant determinant of patients' futures which are supported by the growing body of evidence (Kadthur *et al.*, 2011; Apostolou *et al.*, 2012; Rodrigues *et al.*, 2016).

Another interesting result is the statistically significant correlation between vimentin expression as EMT representative and ALDH1. Although similar correlation has been reported by others in the cell cultures of canine mammary gland cancers (Michishita *et al.*, 2012), this work was the first study that reported this correlation in patients bearing CMGTs. Similarly, Seino *et al.* (2016) and Vieira *et al.* (2020) showed such relation in head and neck malignancy in which high ALDH1 expression was accompanied by EMT, resulting in unfavorable patient outcome. A possible explanation might be that concurrent expression of vimentin and ALDH1 as a CSCs marker could be suggestive of a hybrid potential phenotype with the extreme power of creating metastatic foci. (Floor *et al.*, 2011; Kim *et al.*, 2014; Sulaiman *et al.*, 2019; Savelieva *et al.*, 2020)

No significant correlation was found among TILs and different clinipathologic criteria which are in agreement with Lopes-Neto *et al.* (2021) findings. Despite numerous works in HBC showing prognostic and predictive significance of TILs, there are very limited studies on TILs in canine MMGTs, (Kim *et al.*, 2013; Carvalho *et al.*, 2014; Polónia *et al.*, 2017) that warrants further works with larger sample sizes in the future.

In conclusion, CSCs with high proliferative capacity can result in more aggressive biologic behavior in both canine MMGTs and HBCs. Apart from that, bitches with spontaneous mammary gland tumors seem to be an appropriate model for studying breast cancer complexities regarding CSCs and their critical roles in different hallmarks of malignancy. Although the study has successfully demonstrated such significant findings and opened the way of studying CSCs in canine malignancies in Iran, it has certain limitations in terms of sample size which warrants further studies with greater sample sizes in the future. Furthermore, designing further studies based on multiple CSCs markers and analyzing their molecular pathways could be more helpful in future veterinary oncology and precision target therapies.

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STATEMENT ON COMPETING INTEREST

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

HM, SM, NP, ZA, KGR, and NS have been involved in conceptualization, methodology, and project administration. KV, YGH, SM, ShNL, and HM have been involved in analysis, interpretation of data, and drafting the manuscript. All authors have read and agreed to the final text.

REFERENCES

Ahmadi SA, Moinfar M, Moghaddam KG and Bahadori M. 2010. Practical application of angiogenesis and vasculogenic mimicry in prostatic adenocarcinoma. *Archives of Iranian Medicine* 13(6): 498-503.

Aleskandarany MA, Sonbul SN, Mukherjee A and Rakha EA. 2015. Molecular mechanisms underlying lymphovascular invasion in invasive breast cancer. *Pathobiology* 82(3-4): 113-123.

Andonegui-Elguera MA, Alfaro-Mora Y, Cáceres-Gutiérrez R, Caro-Sánchez CHS, Herrera LA and Díaz-Chávez J. 2020. An Overview of Vasculogenic Mimicry in Breast Cancer. *Frontiers in Oncology* 10: 1-8.

Apostolou P, Toloudi M, Chatziioannou M, Ioannou E and Papasotiriou I. 2012. Cancer stem cells stemness transcription factors expression correlates with breast cancer disease stage. *Current Stem Cell Research & Therapy* 7(6): 415-419.

Ayob AZ and Ramasamy TS. 2018. Cancer stem cells as key drivers of tumour progression. *Journal of Biomedical Science* 25(1): 1-18.

Carvalho MI, Pires I, Prada J and Queiroga FL. 2014. A role for T-lymphocytes in human breast cancer and in canine mammary tumors. *BioMed Research International* 2014: 1-11.

Carvalho MI, Silva-Carvalho R, Pires I, Prada J, Bianchini R, Jensen-Jarolim E and Queiroga FL. 2016. A comparative approachof tumor-associated inflammation in mammary cancer between humans and dogs. *BioMed Research International* 2016: 1-12.

Cassali GD, Lavalle GE, De Nardi AB, Ferreira E, Bertagnolli AC, Estrela-Lima A, Alessi AC, Daleck CR, Salgado BS, Fernandes CG and Sobral RA. 2011. Consensus for the diagnosis, prognosis and treatment of canine mammary tumors. *Brazilian Journal of Veterinary Pathology* 4(2): 153-180.

Caziuc A, Schlanger D, Amarinei G and Dindelegan GC. 2019. Can tumor-infiltrating lymphocytes (TILs) be a predictive factor for lymph nodes status in both early stage and locally advanced breast cancer? *Journal of Clinical Medicine* 8(4): 1-10.

Chaffer CL, San Juan BP, Lim E and Weinberg RA. 2016. EMT, cell plasticity and metastasis. *Cancer and Metastasis Reviews* 35(4): 645-654.

Cidado J, Wong HY, Rosen DM, Cimino-Mathews A, Garay JP, Fessler AG, Rasheed ZA, Hicks J, Cochran RL, Croessmann S and Zabransky DJ. 2016. Ki-67 is required for maintenance of cancer stem cells but not cell proliferation. *Oncotarget* 7(5): 6281-6293.

Cui J, Li P, Liu X, Hu H and Wei W. 2015. Abnormal expression of the Notch and Wnt/ß-catenin signaling pathways in stem-like ALDHhiCD44+ cells correlates highly with Ki-67 expression in breast cancer. *Oncology Letters* 9(4): 1600-1606.

Floor S, Van Staveren WC, Larsimont D, Dumont JE, Maenhaut C. 2011. Cancer cells in epithelial-to-mesenchymal transition and tumor-propagating–cancer stem cells: distinct, overlapping or same populations. *Oncogene* 30(46): 4609-4621.

Kadthur JC, Rao, S, Sonnahallipura BM, Thimmanahalli DS and Laxmikanth SM. 2011. Prognostic Value of Ki 67 proliferation antigen in canine malignant mammary gland tumours. *Brazilian Journal of Veterinary Pathology* 4(1): 36-40.

Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Król M and Jurka P. 2018. Current biomarkers of canine mammary tumors. *Acta Veterinaria Scandinavica* 60(1): 1-13.

Kim ST, Jeong H, Woo OH, Seo JH, Kim A, Lee ES, Shin SW, Kim YH, Kim JS and Park KH. 2013. Tumor-infiltrating lymphocytes, tumor characteristics, and recurrence in patients with early breast cancer. *American Journal of Clinical Oncology* 36(3): 224-231.

Kim YS, Jung MJ, Ryu DW and Lee CH. 2014. Clinicopathologic characteristics of breast cancer stem cells identified on the basis of aldehyde dehydrogenase 1 expression. *Journal of Breast Cancer* 17(2): 121-128.

Lathia J, Liu H and Matei D. 2020. The clinical impact of cancer stem cells. *The Oncologist* 25(2): 123-131.

Lopes-Neto BE, Pinheiro DCSN, Carvalheira JGV, Schmitt F and de Fátima Gärtner M. 2021. Relationship between PD-L1 Expression and Tumor-Infiltrating Lymphocytes in Canine Mammary Tumor. *Acta Scientiae Veterinariae* 49.

Mansour SF and Atwa MM. 2015. Clinicopathological significance of CD133 and ALDH1 cancer stem cell marker expression in invasive ductal breast carcinoma. *Asian Pacific Journal of Cancer Prevention* 16(17): 7491-7496.

Michishita M, Akiyoshi R, Suemizu H, Nakagawa T, Sasaki N, Takemitsu H, Arai T and Takahashi K. 2012. Aldehyde dehydrogenase activity in cancer stem cells from canine mammary carcinoma cell lines. *The Veterinary Journal* 193(2): 508-513.

Mrouj K, Andrés-Sánchez N, Dubra G, Singh P, Sobecki M, Chahar D, Al Ghoul E, Aznar AB, Prieto S, Pirot N, Bernex F. 2021. Ki-67 regulates global gene expression and promotes sequential stages of carcinogenesis. *Proceedings of the National Academy of Sciences* 118(10): 1-12.

Nguyen F, Peña L, Ibisch C, Loussouarn D, Gama A, Rieder N, Belousov A, Campone M and Abadie J. 2018. Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. *Breast Cancer Research and Treatment* 167(3): 635-648.

Paula ADC, Marques O, Sampaio R, Rosa A, Garcia J, Rema A, Faria MDF, Silva P, Vizcaino R and Lopes C. 2016. Characterization of CD44+ ALDH1+ Ki-67– cells in non-malignant and neoplastic lesions of the breast. *Anticancer Research* 36(9): 4629-4638.

Philibert JC, Snyder PW, Glickman N, Glickman LT, Knapp DW and Waters DJ. 2003. Influence of host factors on survival in dogs with malignant mammary gland tumors. *Journal of Veterinary Internal Medicine* 17(1): 102-106.

Polónia A, Pinto R, Cameselle-Teijeiro JF, Schmitt FC and Paredes J. 2017. Prognostic value of stromal tumour infiltrating lymphocytes and programmed cell death-ligand 1 expression in breast cancer. *Journal of clinical pathology* 70(10): 860-867.

Queiroga FL, Raposo T, Carvalho MI, Prada J and Pires I. 2011. Canine mammary tumours as a model to study human breast cancer: most recent findings. *In Vivo* 25(3): 455-465.

Rodrigues H, Carvalho MI, Pires I, Prada J and Queiroga FL. 2016. Clinicopathological significance of caspase-3 and ki-67 expression in canine mammary gland tumours. *Acta Veterinaria Hungarica* 64(1): 78-89.

Rogez B, Pascal Q, Bobillier A, Machuron F, Lagadec C, Tierny D, Le Bourhis X and Chopin V. 2019. CD44 and CD24 expression and prognostic significance in canine mammary tumors. *Veterinary Pathology* 56 (3): 377-388.

Rybicka A and Król M. 2016. Identification and characterization of cancer stem cells in canine mammary tumors. *Acta Veterinaria Scandinavica* 58(1): 1-7.

Saricanbaz I, Karahacioglu E, Ekinci O, Bora H, Kilic D, Akmansu M. 2014. Prognostic significance of expression of CD133 and Ki-67 in gastric cancer. *Asian Pacific Journal of Cancer Prevention* 15(19): 8215-8224.

Savelieva OE, Tashireva LA, Kaigorodova EV, Buzenkova AV, Mukhamedzhanov RK, Grigoryeva ES, Zavyalova MV, Tarabanovskaya NA, Cherdyntseva NV and Perelmuter VM. 2020. Heterogeneity of stemlike circulating tumor cells in invasive breast cancer. *International Journal of Molecular Sciences* 21(8): 2780.

Seino S, Shigeishi H, Hashikata M, Higashikawa K, Tobiume K, Uetsuki R, Ishida Y, Sasaki K, Naruse T, Rahman MZ and Ono S. 2016. CD 44high/ALDH 1high head and neck squamous cell carcinoma cells exhibit mesenchymal characteristics and GSK 3ß-dependent cancer stem cell properties. *Journal of Oral Pathology & Medicine* 45(3): 180-188.

Sulaiman A, McGarry S, Han X, Liu S and Wang L. 2019. CSCs in Breast Cancer—One Size Does Not Fit All: Therapeutic Advances in Targeting Heterogeneous Epithelial and Mesenchymal CSCs. *Cancers* 11(8): 1128.

Vieira RR, Campos LH, Jesus LH, Klabunde C, de Oliveira Gamba T, Flores IL, Oliveira MG and Rados PV. 2020. Overexpression of ALDH1 and EMT marker profile are linked with unfavorable outcome in head and neck cancer. *Medicina Oral, Patología Oral y Cirugía Bucal* 25(6): e752.

Wei H, Fu P, Yao M, Chen Y and Du L. 2016. Breast cancer stem cells phenotype and plasma cell-predominant breast cancer independently indicate poor survival. *Pathology-Research and Practice* 212(4): 294-301.