Evaluation of Cytokeratin 7 Expression in Different Mammary Gland Neoplasms

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Abstract

BACKGROUND: Cytokeratins are non-contractile intermediate filaments engaged in anchoring and structural functions forming a network to support cytoplasm. Cytokeratin 7 (CK7) expression in human breast carcinomas has proved to be a useful differentiation marker, but its expression in canine mammary gland tumors is poorly understood.

OBJECTIVES: Cytokeratin 7 (CK7) expression in human breast carcinomas has proved to be a useful differentiation marker, but its expression in canine mammary gland tumors is poorly understood.

METHODS: This research was based on the immunohistochemical study of CK7 in 17 cases of canine mammary gland neoplasms obtained from the Department of Pathology, Faculty of Veterinary Medicine, University of Tehran. Masson's trichrome staining was performed to differentiate between collagen fibers and smooth muscle.

RESULTS: CK7 protein was detected in both epithelial (1 benign mixed tumor, 1 fibroadenoma, 1 complex carcinoma, and 1 carcinoma mixed type) and myoepithelial (1 fibroadenoma, 1 benign mixed tumor, 3 complex carcinomas, 1 ductal carcinoma, and 1 carcinoma mixed type) cells. Fine and thick collagen fibers were observed in the sections stained by Masson's trichrome.

CONCLUSIONS: Despite using CK7 as a differentiation marker in human breast cancer, CK7 had a controversial expression in the epithelial and myoepithelial cells in canine mammary gland neoplasms. Based on the results, CK7 could not be considered as an independent marker for the canine mammary glands epithelial cell detection and a prognostic factor in canine mammary gland neoplasms.

KEYWORDS: Cytokeratin 7, Dogs, Mammary neoplasms, Masson's trichrome, Myoepithelial cells

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Introduction

One of the most frequently occurring tumors in dogs is the mammary gland tumors (Salas *et al.*, 2015; Pastor *et al.*, 2018; Sharma *et al.*, 2018). Histologically and clinically, there are similarities between canine mammary glands and human breast tissue. Hence, dogs can be considered as in vivo models for the human breast cancer (Cassali, 2013; Abdelmegeed and Mohammed, 2018). The architecture of mammary tissue is composed of three cell populations: luminal epithelial cells, basal/myoepithelial cells, and mesenchymal cells (Sorenmo *et al.*, 2011). The wide heterogeneity of these tumors has increasingly made the classification scheme complex (Schlafer, 2016). In 6 out of 34 histological types of neoplasms, myoepithelial cell proliferation was demonstrated; three of them showed malignant transformation of myoepithelial (Goldschmidt *et al.*, 2011).

Myoepithelial cells are well-developed in canine sweat and mammary glands (Beha *et al.*, 2012; Ingthorsson *et al.*, 2015). In mammary tissue, they form a continuous layer around ducts and teat sinuses (spindle-shaped) and a discontinuous layer around alveoli (star-shaped). They are located between luminal epithelial cells and stromal fibroblasts, which makes them suitable to link with these two cell populations and they have characteristics of muscle and epithelial cells (cytokeratins) (Beha *et al.*, 2012).

Different types of epithelial cells synthesize filaments that are products of co-polymerization of at least one type I and one type II cytokeratin. These filaments which are abundant in the cytoplasm of epithelial cells are called soft keratin. They form intra-cytoplasmic cytoskeleton which causes resistance against mechanical stress (Cooper, 2000). Intermediate filaments are the most resistant filaments against mechanical stress among all filaments that form the cytoskeleton. Cytokeratins are important intermediate filaments that are categorized into six types according to the protein structure. Type I and II are acidic (CK-1, CK-2, CK-3, CK-4, CK-5, CK-6, CK-7, CK-8) and basic (CK-9, CK-10, CK-11, CK-12, CK-13, CK-14, CK-15, CK-16, CK-17, CK-18, CK-19, CK-20) cytokeratins, respectively. Also, according to the molecular weight they are categorized into high molecular weight cytokeratins (squamous keratins) and low molecular weight cytokeratins (simple keratins) (Rekhtman, 2011). They are widely used as epithelial cell markers to assess the tissue origin of cancer (Jacob, 2018). Cytokeratin 7 (CK7) is a type II keratin (basic keratin, low molecular weight) which improves the integrity of cellular structures (Moll *et al.*, 2008). It has been shown that this intermediate filament is expressed in normal canine apocrine glands (Espinosa De Los Monteros *et al.*, 1999). Previously, CK7 expression was subjected to several studies on various canine tissues including cutaneous lesions and mammary glands (Pieper *et al.*, 2015; Eivani and Mortazavi 2016). CK7 is used as a differential diagnosis marker for urothelial tumors, adenocarcinomas, bile duct and lung epithelia (Painter *et al.*, 2010; Pieper *et al.*, 2015; Luo *et al.*, 2017).

The routine staining method of hematoxylin and eosin (H&E) is incapable of distinguishing various eosinophilic tissue components, though special staining techniques such as Masson's trichrome have proved to be cost-effective methods to demonstrate the presence and pattern of collagenous tissue. Despite being old-fashioned, this multi-colored method is still a popular stain for the connective tissue assessment in modern histology (Alturkistani *et al.*, 2016). In this study, this method was used to investigate the increase in collagenous tissue, either as a desmoplasia (stromal cell proliferation) or probable changes in myoepithelial cell nature in the neoplastic process.

The current study aimed to investigate the expression of CK⁷ in canine normal and neoplastic mammary gland epithelial and

myoepithelial cells and to evaluate its association with malignancy grade and ,histologic characteristics of neoplasms. Also Masson's trichrome was chosen to differentiate between smooth muscle fibers and collagen fibers.

Materials and Methods

Samples and Histopathology

This study was based on 17 cases of canine mammary gland neoplasms (n=17) obtained from Department of Pathology, Faculty of Veterinary Medicine, University of Tehran. The samples were fixed in 10% neutral buffered formalin, then cut into 5 μm sections and stained with routine H&E and Masson's trichrome methods. Histopathological types, subtypes, and grades were evaluated according to the Peña method (Peña, 2013) (Table 1). Histopathological variables included histological type and grade of tumors, intra-tumoral necrosis, and lymphovascular invasion.

Table 1. Method for grading. Modification of Pena method for histologic grading of canine mammary cancer

Type of tumor	Criteria for Histological Malignancy Grade			Total scoring (A+B+C)	Grade of Malignancy
	A.Tubule formation (of the specimen)	B.Nuclear pleomorphism	C.Mitoses per 10 HPF		
Benign tumors	0	0	0	0	0
Malignant tumors	> 75% (1)*	Uniform nucleus and occasional nucleoli (1)*	0-9 (1)*	3-5	I
	10-75% (2)*	Moderate variation in nuclear size and shape, hyperchromatic nucleus and presence of nucleoli (2)*	10-19 (2)*	6-7	II
	< 10% (3)*	Marked variation in nuclear size, hyperchromatic nucleus and prominent nucleoli (3)*	>20 (3)*	8-9	III

a * Points

bHPF, high-power field.

Source: Peña, L., De Andres, P.J., *et al.* (2013) 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 50:94–105. Reproduced with permission of SAGE Publications

Masson's Trichrome Staining

After deparaffinization and rehydration, the slides were stained with hematoxylin for 10 min, then Biebrich scarlet-acid fuchsin solution for 2 min. After that they were placed in the phosphomolybdic-phosphotungstic acid solution for 10-15 min and aniline blue solution for 5 min. Finally, the slides were placed in 1% acetic acid for 3-5 min, and then dehydration and clearing were performed.

Antibodies and Immunohistochemistry

Immunohistochemistry for CK7 was performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections at 4 μm diameter. The procedure was performed using ready-to-use

mouse monoclonal antibody (clone OV-TL 12/30 Novocastra Leica Biosystems), which recognizes human CK7 protein. A horseradish peroxidase (HRP) polymer detection method was used for the CK7 antibody. After deparaffinization and rehydration, antigen retrieval was carried out by microwave oven at 750 W and 180 W (citrate buffer, 15 min, pH=6, 0.01 M). To neutralize the endogenous peroxidase, all slides were incubated in hydrogen peroxide 3% for 5 min. To reduce the non-specific bindings, all slides were incubated in protein blocker reagents for 5 min. The slides were incubated with the primary antibody CK7, the post-primary antibody, and Novolink polymer (HRP) Each step for 1 h. Next, the diaminobenzidine (DAB) working solution was used as the chromogen for 10 min. Finally, the slides were counterstained in hematoxylin.

Positive and negative controls were set by adjacent normal mammary tissue and replacing the primary antibody by PBS. The semi-quantitative assessment was done based on the Ramalho *et al.* study (2006) by two observers through randomly chosen 30 fields at high power (×400). Tumors exhibiting brown cytoplasmic staining at >10% neoplastic cells were regarded as positive.

Results

Two out of 17 tumors (11.76%) were benign, and 15 out of 17 were malignant. Among the malignant tumors (n=15), 10 were classified as grade I (66.66%) and 5 were classified as grade II (33.33%). In Masson's trichrome staining, epithelial cells, myoepithelial cells stained red, and the nucleus of fibroblasts, chondroblasts, and osteoblasts were stained dark blue, and collagen fibers, bone, and cartilage matrices were stained blue. No muscular tissue was observed in the tumor sections. Summarized results of the histopathology and immunohistochemical assessments of the examined sections of neoplastic mammary tissues were presented in Table 2.

Table 2. Classification, grading, and immunohistochemical results of canine mammary gland tumors evaluation

Tumor type	Histological type	No. (%)	Grade	Microscopic features		CK7 expression	
				Tumor parenchyma	Tumor stroma	Epithelial	Myoepithelial
Benign (11.76%)	Fibroadenoma	1 (5.88%)	0	Ductal, tubular pattern	Myxoid matrix	±	±
	Benign mixed tumor	1 (5.88%)	0	Tubuloalveola, solid sheet	Desmoplasia, lymphoplasmacytic infiltration	±	±
Malignant (88.23%)	Complex carcinoma	7 (41.17%)	I	Ductal, intraductal papillary pattern,	Desmoplasia, lymphoplasmacytic infiltration	- (41.17%)	- (n=5, 29.41%) ± (n=2, 11.76%)
		1 (5.88%)	II	chondromucinous appearance, tubuloalveolar		± (5.88%)	+ (n=1, 5.88%)

Tumor type	Histological type	No. (%)	Grade	Microscopic features		CK7 expression	
				Tumor parenchyma	Tumor stroma	Epithelial	Myoepithelial
				with tubulopapillary pattern			
	Ductal carcinoma*	2 (11.76%)	II	Ductal, papillary pattern, chondromucinous appearance	Desmoplasia, mixed inflammatory infiltration	- (n=2)	- (n=1, 5.88%)
							+ (n=1, 5.88%)
	Carcinoma mixed type	1 (5.88%)	II	Ductal, tubular pattern	Myxoid matrix, lymphoplasmacytic infiltration, chondroid metaplasia	+	-
		1 (5.88%)	I	Tubuloalveolar, tubular pattern		-	+
	Carcinosarcoma	2 (11.76%)	I	Tubuloalveola, tubulopapillary pattern*	Hemangiopericytoma-like pattern, chondroid metaplasia, bone metaplasia	-	-
	Carcinoma and malignant myoepithelioma	1 (5.88%)	II	Tubuloalveola, tubular pattern	Desmoplasia, myxoid matrix	-	-

a *Local invasion

b numbers in the brackets are the relative frequencies.

H&E and Connective Tissue Staining Findings

Benign Neoplasms

The proliferation of ducts was observed in a hypocellular stroma in fibroadenoma and desmoplasia in the benign mixed tumor. In Masson's trichrome staining, the nucleus of spindle and stellate stromal fibroblasts were demonstrated as dark blue, and the thick collagen fibers of fibroadenoma and benign mixed tumor were demonstrated as blue (Figure 1).

Malignant Neoplasms

The malignant neoplasms are divided into malignant epithelial neoplasms and mixed neoplasms. In complex carcinomas (n=8) (malignant neoplasms), the proliferation of both epithelial and myoepithelial cells, areas of myxoid matrix, cholesterol cleft, and lipofuscin laden macrophages were observed. In one ductal carcinoma, areas of squamous differentiation, foci of necrosis, myxoid matrix, cholesterol cleft, and mixed inflammatory infiltration were observed. In carcinoma and malignant myoepithelioma, nests and trabeculae of epithelial cells and malignant proliferation of myoepithelial cells with scant myxoid matrix, necrosis, cholesterol cleft, desmoplasia, and lipofuscin pigments were observed. Periductal, interlobular, and intralobular collagen fibers were demonstrated

as blue fibers in Masson's trichrome stain ([Figure 1. A & D](#)). This regular pattern was observed in 8 complex carcinomas, 2 ductal carcinomas, and 1 carcinoma and malignant myoepithelioma. In some tumors, intratumoral collagen fibers were recognized as fine distinct fibers interspersed between proliferative normal and malignant epithelial and myoepithelial cells (1 benign mixed tumor, 8 complex carcinomas, 1 carcinoma and malignant myoepithelioma) ([Figure 1](#)).
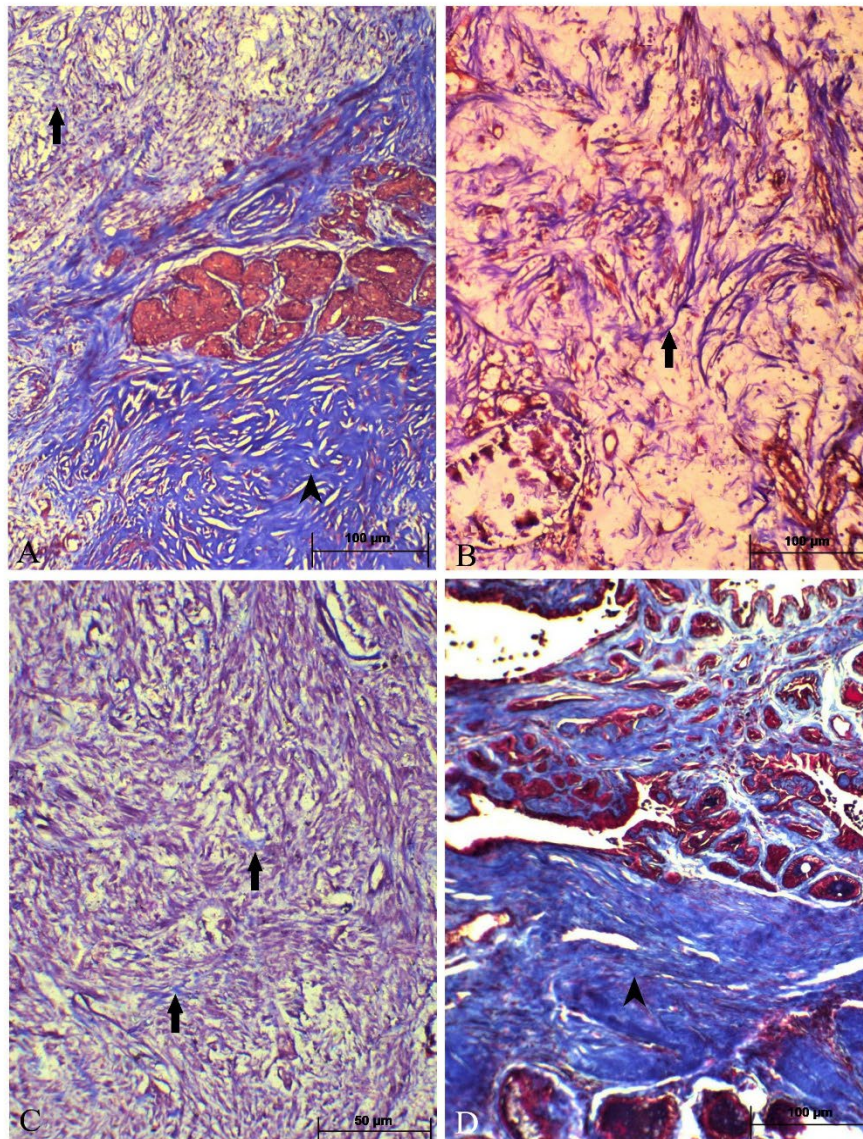

Figure 1. Canine mammary gland neoplasia. Intratumoral fine (arrows) and thick (pointed arrows) collagen fibers. Masson's Trichrome. (A & B. Complex carcinoma, 100×, C. Carcinoma and malignant myoepithelioma, 200× & D. Fibroadenoma, 100×).

In carcinoma mixed type (mixed neoplasms), foci of chondroid metaplasia with the proliferation of epithelial cells were observed. In one carcinosarcoma proliferation of epithelial cells, the malignant hemangiopericytoma-like proliferation pattern of stromal fibroblasts and areas of chondroid metaplasia and bone metaplasia were observed. In Masson's trichrome staining, collagen fibers were recognized as fine distinct

fibers in carcinoma mixed type and interlobular and intralobular fibers in carcinosarcomas.

CK7 Immunoreactivity

CK7-labeled epithelial cells were observed in a normal case and four cases of tumors [23.53%] (1 benign mixed tumor, 1 fibroadenoma, 1 complex carcinoma, and 1 carcinoma mixed type) (Figure 2. A & E) and myoepithelial cells in seven cases [41.17%] (1 fibroadenoma, 1 benign mixed tumor, 3 complex carcinomas, 1 ductal carcinoma, and 1 carcinoma mixed type) (Figure 2. C & F). Negative labeling of epithelial (Figure 2. B, D, & F) and myoepithelial (Figure 2. A, B, D, & E) cells were observed in 13 (76.47%) and 10 (58.82%) tumors, respectively (Table 2).
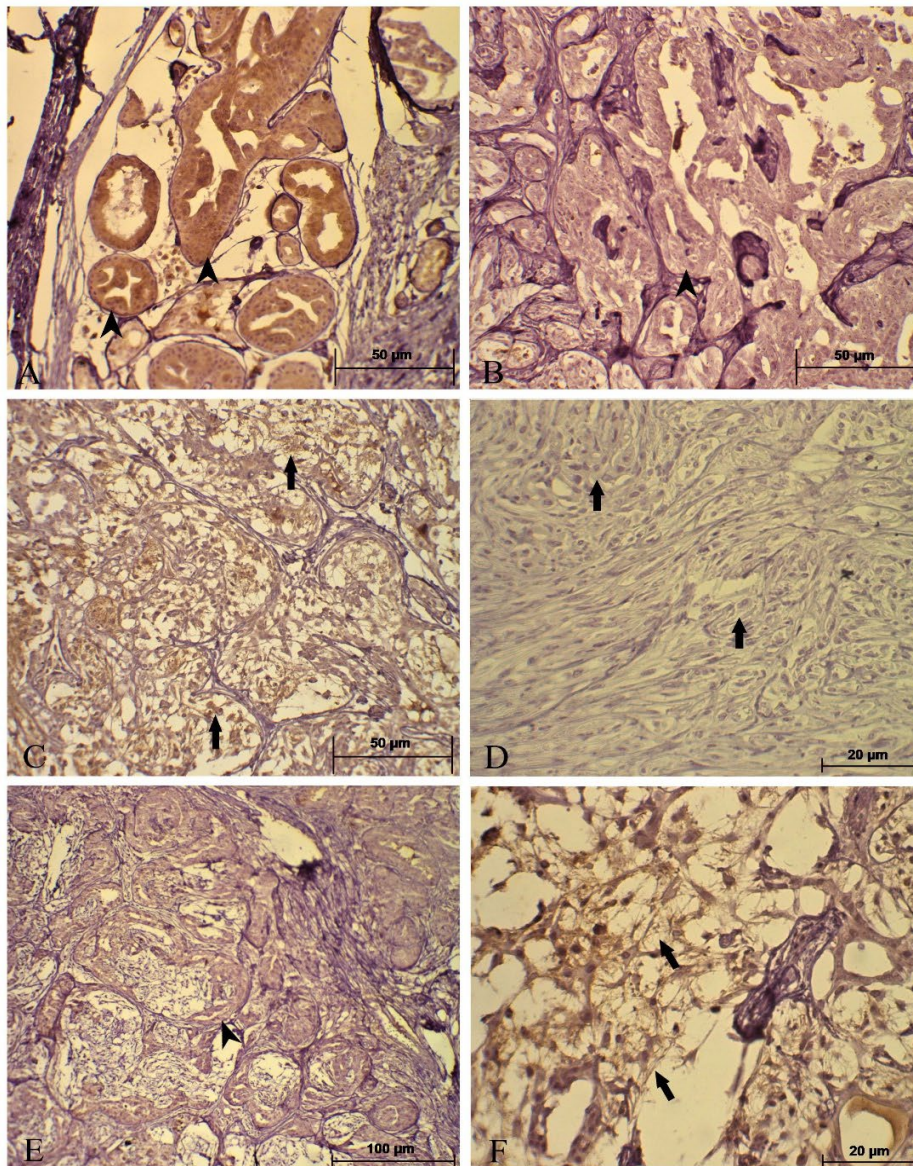

Figure 2. Canine mammary gland carcinoma. Note positive immunolabeling of CK7 in epithelial cells (A, 200× & E, 100×) and myoepithelial cells (C, 200× & F, 400×) and negative immunolabeling of CK7 in epithelial cells (B, 200× & F) and myoepithelial cells (A, B & D, 400×). Proliferative myoepithelial cells stained positive. (Epithelial cells: Pointed arrows, myoepithelial cells: Arrows, A, B & E. Complex carcinoma, C & F. Carcinoma mixed type, D. Carcinoma and malignant myoepithelioma). C & F are from the same samples.

Discussion

Tumor studies could provide advantages in terms of the etiology, development, and treatment of cancer. Among all domesticated species, dog is more susceptible to tumor progression because of serving as a companion animal to humans and exposure to the same predisposing factors as humans (Salas *et al.*, 2015; Baioni *et al.*, 2017; Pastor *et al.*, 2018). In veterinary medicine, several studies have focused on normal canine mammary gland histology and its morphological changes through the estrous cycle. There is some evidence to suggest that the mammary glands undergo various stages of growth (development and regression), depending on its hormonal environment (Santos *et al.*, 2010; Sorenmo *et al.*, 2011). Hence, it is not surprising that mammary neoplasms have a higher incidence in intact female dogs than in male dogs (Salas *et al.*, 2015; Baioni *et al.*, 2017).

Epithelial and myoepithelial tumors are dominant tumors of the mammary glands (Schlafer, 2016). The origin of these cell populations has not been elucidated, but a stem/progenitor cell and bipotent mammary precursors have been suggested (Visvader and Stingl, 2014). In mammary glands, epithelial and myoepithelial cells may have a polygonal morphology (Beha *et al.*, 2012).

In the present study, the ratio of benign-to-malignant neoplasms was obtained 2:15. Various studies have reported different ratios (Baioni *et al.*, 2017; Gabli *et al.*, 2017). Conversely, in women, most breast masses detected on clinical examinations are categorized as benign breast disease. In this research, complex carcinomas had the highest number (8:17) among all histological types ([Table 2]). Peña *et al.* (2013) and Santos *et al.* (2013) reported complex carcinoma as the most frequent neoplasm of their studies (Peña *et al.*, 2013; Santos *et al.*, 2013).

In different neoplasms, epithelial cells reacted differently to CK7 antibody. In benign neoplasms and hyperplastic glands, epithelial cells mainly reacted with the CK7 antibody. Proliferative myoepithelial cells reacted positively to the CK7 antibody. This different immunoreactivity to CK7 antibody suggests that these cells have followed different paths in their keratin maturation or expression. Another possible explanation is related to the CK7 content in the epithelial and myoepithelial cells. Since CK7 content is low, it is not detectable until after proliferation or neoplastic transformation. The positive reactivity for CK7 in elongated myoepithelial cells could be indicative of gaining CK7 expression during progression toward chondromucinous tissue by these cells.

It has been postulated that stellate motile myoepithelial cells change into fibroblasts due to the discontinuous labeling of Alpha-SMA, loss of CK14, CK5/6, and p63 expression (myoepithelial suprabasal markers) and retention of an affinity for vimentin (Ramalho *et al.*, 2006).

Recent studies have focused on finding a trusted marker or panel of markers for myoepithelial cells, but none of them enjoyed 100% sensitivity and specificity, though p63 and CK14 have been recommended as suitable markers for myoepithelial cells (Gama *et al.*, 2003; Beha *et al.*, 2012; Moritani *et al.*, 2015).

There is a growing body of evidence that is changing our point of view toward carcinogenesis to a more complicated process consisting of interactions between epithelial, myoepithelial, and mesenchymal (mainly extracellular matrix) elements (Sirka *et al.*, 2018). Myoepithelial cells in the earliest stage of proliferation, form spherical masses in which cells with elongated to stellate nuclei are trapped in a slightly basophilic ground substance (chondromucinous appearance) (Moulton, 1978). In this stage,

alkaline phosphatase reactivity is diminished. This process suggests that this phenomenon is caused by the collapse of the morphostat gradient and disruption of the tissue architecture (extracellular matrix) in a prolonged process (Baker *et al.*, 2010). Similarly, the negative results of CK7 labeling in some tumors could be the result of this process which changes the immunoprofile of myoepithelial cells – «converted myoepithelial cells» (Baker *et al.*, 2010). Regarding different benign to malignant tumor ratios in humans and dogs, authors suggest that different stages of neoplastic cell differentiation could affect differentiation marker expression and its expression pattern.

Masson's trichrome special stain was used to differentiate between smooth muscle cells and fibrous connective tissue. We did not observe smooth muscle cells in any of the samples.

The most notable observation in this study was the negative reaction of some epithelial cells to CK۷ antibody in canine mammary gland neoplasms. Considering only the expression or lack of expression, CK۷ expression is not significantly associated with tumor type and grade. Asimilar observation was reported by Eivani and Mortazavi, (۲۰۱٦). Despite using cytokeratins as epithelial cell marker in human breast cancer, their role in canine mammary gland tumor prognosis is not clear.

Conclusion

In conclusion, CK۷ could not be considered as an independent marker for the canine mammary glands epithelial cell detection and a prognostic factor in canine mammary gland neoplasms, contrary to human.

Acknowledgments

Conflict of Interest

Authors declared no conflicts of interest.

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ارزیابی بیان سیتوکراتین ۷ در نئوپلازی‌های مختلف غدد پستانی

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زمینه مطالعه: سیتوکراتین‌ها فیلامان‌های بینابینی غیرانقباضی‌اند که در فعالیت‌های ساختمانی و اتصالی که در تشکیل شبکه‌ای برای حفاظت از سیتوپلاسم دخالت دارند. بیان سیتوکراتین ۷ در کارسینوم‌های پستان انسان مارکر مفیدی برای تمایز است اما بیان ان در تومورهای پستانی سگ به‌خوبی شناخته نشده است.

هدف: در بررسی تفکیک و تمایز تومور های پستانی در انسان بیومارکر CK7 نقش مفیدی دارد ولی نقش آن در تومور های پستانی سگ نا مشخص است.

روش کار: این مطالعه بر اساس مطالعه ایمنوهیستوشیمیایی سیتوکراتین ۷ در ۱۷ مورد نئوپلاسم پستان سگ اخذشده از گروه آسیب‌شناسی دانشکده دامپزشکی دانشگاه تهران و رنگ‌آمیزی تری‌کروم ماسون نمونه‌های مذکور برای نشان دادن رشته‌های کلاژن انجام شده است.

نتایج: پروتئین سیتوکراتین ۷ در هر دو سلول‌های اپیتلیالی (۱ تومور مختلط خوش‌خیم، ۱ فیبروآدنوما، ۱ کارسینوم کمپلکس، ۱۱ کارسینوم مختلط) و میواپیتلیالی (۱ فیبروآدنوم، ۱ تومور مختلط خوش‌خیم، ۳ کارسینوم کمپلکس، ۱ کارسینوم مجرایی و ۱ کارسینوم مختلط) شناسایی شده است. رشته‌های کلاژن باریک و ضخیم در مقاطع رنگ‌آمیزی‌شده با تری‌کروم ماسون مشاهده شده است.

نتیجه‌گیری نهایی: با وجود استفاده از سیتوکراتین ۷ به‌عنوان مارکر تمایزی در سرطان پستان انسان، در بافت پستان سگ، سیتوکراتین ۷ بیان بحث‌برانگیز در سلول‌های اپیتلیالی و میواپیتلیالی داشت. بر اساس نتایج فوق، سیتوکراتین ۷ نمی‌تواند به‌عنوان مارکر مستقل برای شناسایی سلول‌های اپیتلیالی غدۀ پستانی سگ و فاکتور پیش‌آگهی‌دهنده در نئوپلاسم‌های پستان سگ در نظر گرفته شود.

واژه‌های کلیدی: ری‌کروم ماسون، سگ، سلول میواپیتلیال، سیتوکراتین ۷، نئوپلاسم پستان

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