Research in Veterinary Science 140 (2021) 56–63

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Expression of VCAN and its receptors in canine mammary carcinomas with or without myoepithelial proliferation

S.H. Figuerêdo [a], R.S. Carmo Neto [b], E. Ferreira [c], G.D. Cassali [c], A. Estrela-Lima [b], K. A. Damasceno [a,*]

[a] Laboratory of Experimental Pathology, Gonçalo Moniz Institute, Oswaldo Cruz Foundation, 121 Rua Waldemar Falcão., Salvador BA 40296-710, Brazil
[b] Department of Pathology and Clinics, School of Veterinary Medicine and Zootechny, Federal University of Bahia, s/n° Adhemar de Barros., Salvador BA 40170-110, Brazil
[c] Department of General Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, 6627 Av. Pres. Antônio Carlos, Belo Horizonte MG 31270-901, Brazil

ARTICLE INFO

Keywords:
Canine
Cancer progression
Extracellular matrix
Versican

ABSTRACT

The proteoglycan versican (VCAN) plays a complex role in cancer. The expression of this molecule has been related to invasion and progression in malignant mixed tumors, such as carcinoma in mixed tumors (CMT) of the canine mammary gland. In addition, its interaction with surface cell receptors EGFR, HER-2 and CD44 in malignant epithelial cells may be responsible for proliferation and cellular motility in early stages of cancer. We comparatively evaluated the expression of this proteoglycan and its receptors in *in situ* and invasive areas of simple carcinomas (SC) and CMT to investigate similarities and differences between these histological types. Immunohistochemistry was performed with anti-VCAN, anti-CD44, anti-EGFR and anti-HER-2 antibodies in 32 cases of SC or CMT. VCAN was highly expressed in stroma adjacent to invasive areas in SC and CMT. CMTs presented comparatively higher expression of VCAN in stroma adjacent to *in situ* and in invasive areas than in corresponding areas in SCs. In CMT, EGFR and HER-2 expressions were higher *in situ* compared to invasive areas. In contrast, increased CD44 and EGFR expression was found in invasive areas in SC compared to CMT. These results indicate that versican expression is similarly associated with invasiveness in SC and CMT, however higher levels were seen in CMT suggesting that the presence of myoepithelial proliferation in this tumor type participates in stromal composition and promoting an increase in the expression of versican.

1. Introduction

Increasing evidence indicates that molecules in the extracellular matrix (ECM) are involved in direct signaling, either through interactions with receptors, such as integrins, or with growth factor receptors (Rutnam et al., 2013). Specifically, proteoglycans play an important role in adherence interactions occurring in the ECM and on cell surfaces. Versican (also known as CSPG2 or VCAN), a chondroitin sulfate proteoglycan, exhibits anti-adhesive properties and is capable of modulating cell proliferation and migration (Du et al., 2013), promoting tumor progression, proliferation, cell differentiation and angiogenesis (Kischel et al., 2010).

Associations between the overexpression of versican and neoplastic cell invasion have been demonstrated in malignant epithelial tumors in women (Skandalis et al., 2011) and canines (Erdélyi et al., 2003; Damasceno et al., 2012). The binding of versican with hyaluronic acid, as well as its interactions with CD44 cellular surface receptors and epidermal growth factor receptor (EGFR), leads to the formation of a complex capable of triggering intracellular signaling mechanisms that is believed to promote cancer cell motility through the regulation of neoplastic cell proliferation and migration in the ECM (Hernández et al., 2011).

Similarly to humans, mammary carcinoma is the most frequent type of cancer occurring among canine females (Bray et al., 2018; Uva et al., 2009). Among the malignant mammary tumors that occur in female dogs, carcinomas in mixed tumors (CMT) are the most frequent (Cassali et al., 2011; Cassali et al., 2017; Nunes et al., 2018). This neoplasia is characterized by the malignant of epithelial cells, in addition to the presence of myoepithelial and mesenchymal (chondroid and/or osteoid) components. The proliferation of myoepithelial cells, and the

* Corresponding author.
E-mail address: karine.damasceno@fiocruz.br (K.A. Damasceno).

https://doi.org/10.1016/j.rvsc.2021.08.010
Received 9 December 2020; Received in revised form 13 July 2021; Accepted 6 August 2021
Available online 8 August 2021

production of the myxoid matrix by these same cells, are both important to the diagnosis of this tumor type. Accordingly, the phenotypic evaluation of myoepithelial cells and ECM components has become the object of studies attempting to elucidate mechanisms involved in the biological behavior of these tumors (Erdélyi et al., 2003; 2005).

Unlike CMT, simple carcinomas (SC) are characterized by the epithelial proliferation without myoepithelial cells and mesenchymal components. This tumor type, among the least frequent tumors occurring in canine females, presents solid, tubular or papillary subtypes (Cassali et al., 2014; Goldschmidt et al., 2011). Morphologically, solid carcinomas are characterized by low amounts of intratumor stroma around islands of neoplastic cells that form solid arrangements. Tubular carcinomas present intense desmoplasia adjacent to groups of malignant epithelial cells arranged in tubule-like structures. Both subtypes are highly invasive and considered to be medium or highly aggressive. Papillary carcinomas present moderate amounts of intratumor stroma located on the papillary carcinomatous proliferation axis or adjacent to the carcinoma. These tumors tend to have a favorable prognosis (Nunes et al., 2018).

Our group previously described the overexpression of VCAN in peritumoral stroma adjacent to invasive areas of benign mixed tumors, carcinoma in mixed tumors and carcinosarcomas (Damasceno et al., 2016b). Significant differences in the expression of this proteoglycan among these three histological tumor types were described, as well as in this molecule's membrane receptor expression. Based on these previous findings, it is relevant to understand the differences in the expression of VCAN and its receptors in tumors without myoepithelial proliferation, such as simple carcinomas.

This study aimed to comparatively evaluate versican expression and its possible associations between cell surface receptors in simple carcinomas and carcinomas in mixed tumors of the canine mammary gland. These findings should contribute to the understanding of the dynamics occurring in the extracellular matrix during tumor progression.

2. Materials and methods

2.1. Mammary tumor samples

Mammary carcinoma samples ($n = 32$) were selected of which 17 cases as SC (invasive papillary carcinoma (10/17) (Fig. 1a and b), tubular carcinoma (6/17) and solid carcinoma (1/17)) and 15 cases were diagnosed as CMT (Fig. 2a and b). A retrospective analysis was performed of canine mammary carcinoma cases previously submitted to therapeutic mastectomy, regardless of breed or age. Radiography was performed in three views (right lateral, left lateral and ventral dorsal) and abdominal ultrasound to confirm the presence of metastases.

2.2. Tumor staging

Clinical and pathological staging was performed based on tumor size (T), neoplastic involvement of regional lymph nodes (N) and the presence of distant metastases (M), according to the TNM system established by the World Health Organization (WHO) for canine mammary neoplasms modified from Owen (1980). These data were obtained from the clinical-pathological records of each canine patient.

2.3. Histopathological analysis

Histological sections measuring 4 μm were prepared from selected blocks and stained using the hematoxylin-eosin method. The histological identification of tumor type was performed by three veterinary pathologists and the diagnosis with the highest agreement was considered following the classification criteria of the World Health Organization (WHO) and the Consensus for the Diagnosis, Prognosis and Treatment of Canine Mammary Tumors (Cassali et al., 2014). All tumors were graded according to the Nottingham System (Elston and Ellis, 1998). In addition, the Nottingham Prognostic Index (NPI) was also calculated according to nodule size (cm), lymph node score and the histological grading defined by the Nottingham system. NPI values were used to distribute the cases into three groups: < 3.4 (good prognosis); 3.41–5.4 (moderate prognosis) and > 5.41 (poor prognosis).

2.4. Immunohistochemistry

The primary antibodies used for analysis were versican (12C5, DSHB, 1:50), CD44 (IM7, Santa Cruz, 1:200), EGFR (31G7, Invitrogen, 1:100) and HER-2 (polyclonal, Dako, 1:200). All slides were deparaffinized and rehydrated in a graded ethanol series. For EGFR, CD44, HER-2 and versican staining, the slides were performed antigen retrieval using protocol described by Damasceno et al. (2016). It was used a polymeric system for antibody detection (Novolink™ Max PolymerDetection System). Finally, the sections were exposed to chromogen 3,3 – diaminobenzidine (DAB) and counter-stained with Mayer's hematoxylin. Previously tested mammary gland samples were used as positive controls, while primary antibodies were replaced by normal serum (IgG) for negative controls. All histological assessments were performed under a conventional light microscope (Olympus–BX41) with a 20×, 40× or 60× magnification.

2.5. Immunohistochemical analysis

The expression of versican was evaluated in according to Damasceno et al. (2012). The expression of versican was evaluated using a semi-quantitative scale in adjacent peritumoral stroma, both *in situ* and in invasive areas of malignant epithelial cell proliferation, considering (i) the total percentage of positively stained tissue (0–100%) and (ii) the staining intensity for the proteoglycan. According to the scale, (1) was considered negative or barely stained, (2) weak, (3) moderate and (4) strong. The immunohistochemical score of versican expression was calculated by multiplying the percentage of tissue stained (0–100%) by staining intensity (1–4). Semiquantitative analysis was also performed to determine CD44 expression in areas of malignant epithelial proliferation (*in situ* and invasive). The scale (0) was considered unmarked; (1) poor; (2) moderate; (3) intense (Paltian et al., 2009). With regard to the number of stained cells, (0) indicated the area was negative (1) between 1 and 25% of positive cells; (2) 26–50% of positive cells; (3) 51–75% of positive cells. EGFR and HER-2 expression were analyzed according to the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) (Wolff et al., 2013). Cases were considered positive when complete and intense membrane staining was observed in at least 10% of the malignant epithelial cells.

2.6. Statistical analysis

All analyses were performed with the aid of GraphPad Prism v. 5.0 software (GraphPad, San Diego, CA, USA). Quantitative results with a normal distribution were submitted to variance analysis under 5% probability, followed by means testing. The Chi-square test or Fisher's Exact Method were used for comparisons among variables. Possible correlations were evaluated by Spearman's or Pearson's testing. Significance was considered when $p < 0.05$.

3. Results

3.1. Clinical-pathological characteristics

The mean age at time of CMT diagnosis was 9.93 years, *versus* 12.05 years for SC ($p = 0.0546$). Most CMTs measured between 3 and 5 cm (46.6%), while most SC were ≤ 3 cm (76.5%). Lymph node metastasis was not present in the majority of samples in each group ($p = 0.6586$), nor was distant metastasis ($p > 0.9999$). CMT staging was determined to be II in most cases (46.6%) *versus* I in SCs (64.7%) ($p = 0.2682$).

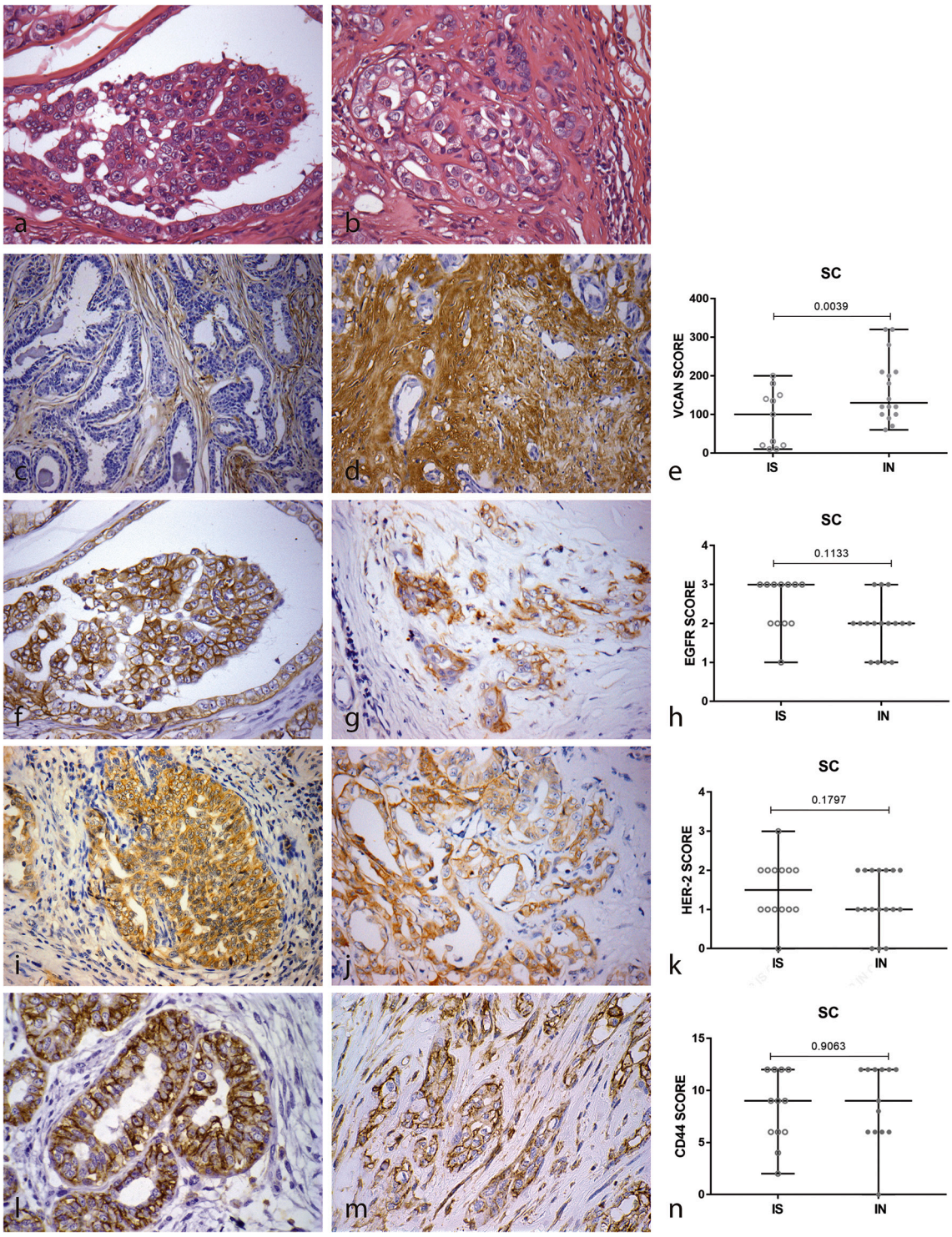

Fig. 1. Immunohistochemical analysis in *in situ* and invasive areas of simple carcinoma (A) Papillary arrangement in carcinomatous areas *in situ*. Epithelial cells reveal moderate pleomorphism characterized by the eosinophilic cytoplasm, round and ovoid nucleus, fragmented chromatin and prominent nucleoli. 400x. (B) Invasive carcinomatous areas characterized by high pleomorphism, clear eosinophilic cytoplasm, round and ovoid nucleus, fragmented chromatin and prominent nucleoli. 400x. (C) Low expression of VCAN in stroma adjacent to *in situ* carcinomatous cells. 200x. (D) VCAN overexpression in stroma adjacent to invasive carcinomatous cells. 200x. (E) The graph shows difference of VCAN expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (F) Complete membrane staining for EGFR (score 3) in *in situ* carcinomatous cells. 600x. (G) Incomplete membrane staining for EGFR (score 2) in invasive carcinomatous cells (arrows). 600x. (H) The graph shows the comparison of EGFR expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (I) Incomplete membrane staining for HER-2 in *in situ* carcinomatous cells (score 2). 400x (J) Incomplete membrane staining for HER-2 in invasive carcinomatous cells (score 2). 600x. (K) The graph shows the comparison of HER-2 expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (L) Strong cell membrane staining for CD44 in *in situ* carcinomatous cells. 600x. (M) Moderate CD44 expression in invasive carcinomatous cells. 600x. (N) The graph shows the comparison of CD44 expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (C,D,F,G,I,J,L, M) Immunohistochemical staining with DAB followed by counterstaining with Mayer's hematoxylin. Legend: VCAN - versican; IS - *in situ* stromal areas; IN - stroma adjacent to invasive areas; SC - simple carcinoma; CMT - Carcinoma in mixed tumor.

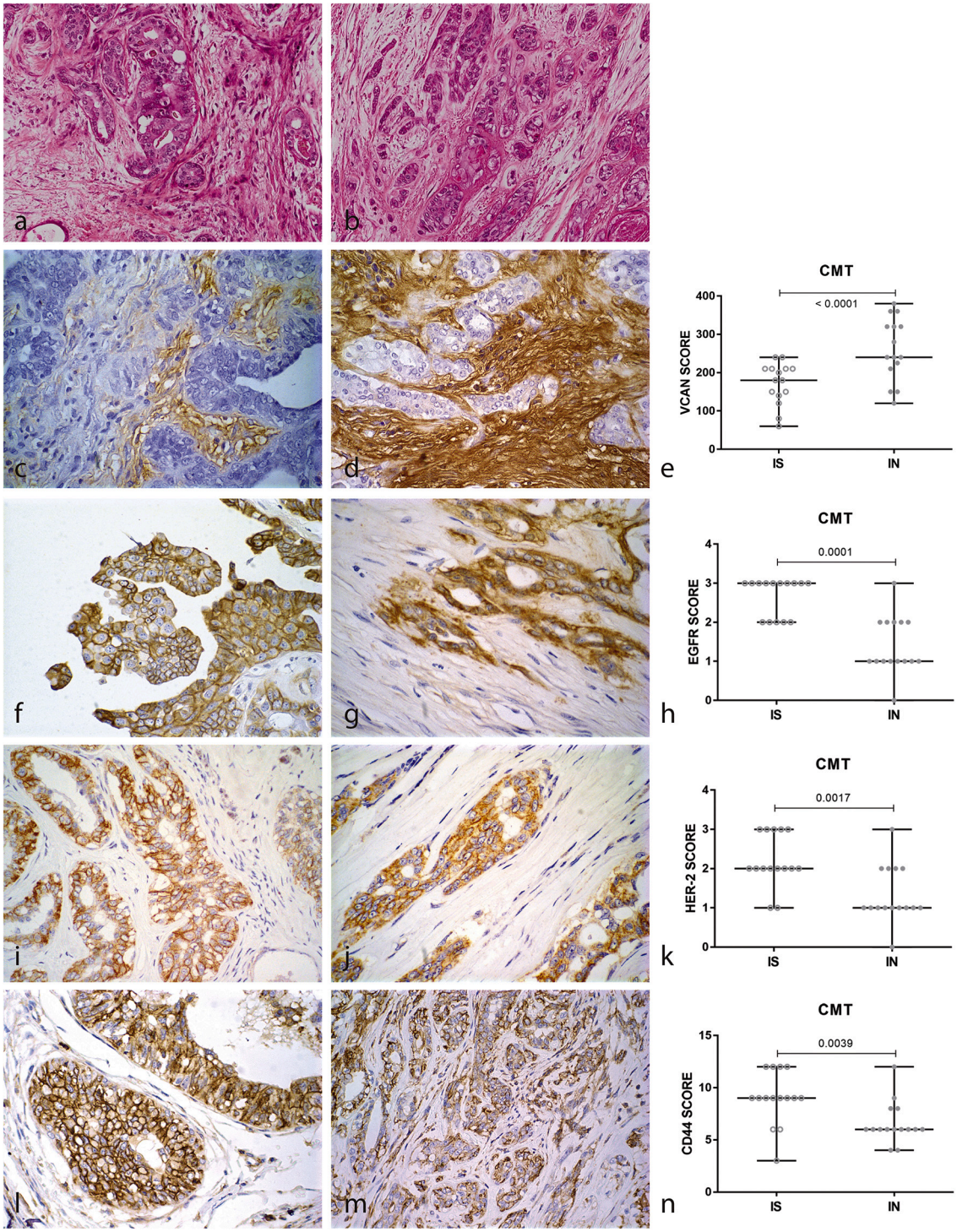

Fig. 2. Histological staining for VCAN, EGFR, HER-2 and CD44 in *in situ* and invasive carcinomatous areas of canine carcinoma in mixed tumor (A) Carcinomatous areas *in situ* characterized by epithelial cells with moderate pleomorphism, eosinophilic cytoplasm, round and ovoid nuclei and fragmented chromatin. Hematoxylin and Eosin staining, 400x. (B) Carcinomatous cells in tubular arrangements with invasion into adjacent stroma. 400x. (C) Weak expression of versican in intratumoral stroma adjacent to carcinomatous *in situ* areas. 400x. (D) Versican overexpression in stroma adjacent to invasive cells. 400x. (E) The graph shows difference of VCAN expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (F) Complete membrane staining for EGFR (score 3) in *in situ* papillary carcinomatous cells. 600x. (G) Incomplete membrane EGFR staining (score 2) in invasive carcinomatous cells. 600x. (H) The graph shows difference of EGFR expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (I) Incomplete membrane HER-2 staining in *in situ* carcinomatous cells (score 2). 400x. (J) Incomplete membrane staining for HER-2 in invasive carcinomatous cells (score 1). 600×. (K) The graph shows difference of HER-2 expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (L) Complete membrane staining for CD44 in carcinomatous cells *in situ* (high expression). 400×. (M) Incomplete membrane staining for CD44 in invasive carcinomatous cells (low expression). 400x. (N) The graph shows difference of CD44 expression between *in situ* and invasive areas. Wilcoxon's test, 95% confidence interval ($p < 0.05$). (C,D,F,G,I,J,L,M) Immunohistochemical staining with DAB followed by counterstaining with Mayer's hematoxylin.

Histological grade I was more frequent in most CMTs (73.3%) as well as in SCs (64.7%) ($p = 0.5745$). Detailed clinical pathological characteristics are described in Table 1.

3.2. VCAN is overexpressed in stroma adjacent to invasive carcinomatous areas in SC and CMT

The median expression of VCAN in stroma adjacent to *in situ* stromal areas was 100 in SC, while in invasive areas a median 190 was obtained in this tumor type($p = 0.0039$) (Fig. 1c, d and e). Similarly, lower VCAN expression was also observed in the stroma adjacent to *in situ* carcinomatous areas (median 180) in CMTs compared to invasive carcinomatous areas (median 240) ($p < 0.0001$) (Fig. 2c, d and e). CMT presented significantly higher VCAN expression compared to SC in both in *in situ* areas ($p = 0.0041$) and stroma adjacent to invasive areas ($p = 0.0022$) (Fig. 3a and b, respectively).

3.3. Higher expression of EGFR and CD44 in invasive carcinomatous areas, and lower HER-2 expression in in situ carcinomatous areas of SC compared to CMT

In *in situ* carcinomatous areas of SC, EGFR expression was scored as 3 in 41.2% of the cases, while 52.9% of these samples received a score of 2 in invasive carcinomatous areas (Fig. 1f, g and h). In CMT, 66.7% of the cases presented a score of 3 in *in situ* carcinomatous areas, while 53.3% of the cases were scored as 1 in invasive carcinomatous areas, with a significant difference between the areas analyzed ($p = 0.0001$) (Fig. 2f, g and h). In addition, higher EGFR expression was seen in invasive areas in SC compared to CMT ($p = 0.0469$) (Fig. 3c).

HER-2 expression in *in situ* areas of SC was predominantly scored as 1 (35.3%) and 2 (35.3%), similarly to invasive areas (41.2% for scores 1 and 2) (Fig. 1i, j and k). In CMTs, 53.3% of the cases revealed a score of 2 in *in situ* areas, which was significantly different than in invasive areas, in which 60% of the samples presented score of 1 ($p = 0.0017$) (Fig. 2i, j and k). Stronger HER-2 expression was seen in *in situ* carcinomatous areas of CMT than SC ($p = 0.016$) (Fig. 3d).

Moderate, high and very high expression of CD44 was detected in 23.5%, 17.6% and 23.5% of the *in situ* carcinomatous areas of SCs, respectively. With regard to invasive carcinomatous areas, 5.9% presented low expression, while 23.5% were moderate, 11.8% high and 23.5% had very high expression (Fig. 1l, m and n). In CMTs, most *in situ* carcinomatous areas presented high expression (53.3; 8/15), which was significantly different than the predominantly moderate expression (73.3%; 11/15) seen in invasive carcinomatous areas ($p = 0.0039$) (Fig. 2l, m and n). In addition, higher CD44 expression was observed in invasive areas of SC cases compared to CMT ($p = 0.047$) (Fig. 3e). Table 2 lists all frequencies found for VCAN, EGFR, CD44 and HER-2 expression in *in situ* and invasive carcinomatous areas of SC and CMT.

The correlation analysis between VCAN and the receptors analyzed in SC revealed that the levels of VCAN expressionlevels of VCAN correlated positively with CD44 expression in *in situ* areas ($p = 0002$; $r = 0.86$). EGFR expression in *in situ* areas of CMT was also positively correlated with VCAN expression in stroma adjacent to both carcinomatous areas (*in situ*, $p = 0.0007$, $r = 0.81$; invasive, $p = 0.0043$, $r = 0.72$).

Table 1
Frequencies of clinical and pathological characteristics in simple carcinomas and carcinomas in mixed tumors of the canine mammary gland.

Clinical pathological characteristics	SC ($n = 17$)	CMT ($n = 15$)	*P valuer
	n/total (%)	n/total (%)	
Mean age (years)	12.05	9.93	0.0546
Tumor size			
≤3 cm	13/17 (76.5)	4/15 (26.6)	0.0388
3 < x ≤ 5 cm	1/17 (5.9)	7/15 (46.6)	
>5 cm	3/17 (17.6)	3/15 (20)	
Lymph node metastasis			
Positive	4/17 (23.5)	2/15 (13.3)	0.6586
Negative	13/17 (76.5)	13/15 (86.6)	
Metastasis			
Positive	0/17 (0)	0/15 (0)	>0.9999
Negative	17/17 (100)	15/15 (100)	
Clinical stage			
I	11/17 (64.7)	3/15 (20)	0.2682
II	0/17 (0)	7/15 (46.6)	
III	2/17 (11.8)	2/15 (13.3)	
IV	4/17 (23.5)	2/15 (13.3)	
V	0/17 (0)	0/15 (0)	
Histological grade			
I	11/17 (64.7)	11/15 (73.3)	0.5745
II	3/17 (17.6)	3/15 (20)	
III	3/17 (17.6)	1/15 (6.6)	

Mann-Whitney test, 95% confidence interval ($p < 0.05$). (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.0001$.

4. Discussion

Our work demonstrated relationships between VCAN expression and tumor invasiveness. In addition, the associations investigated between VCAN and EGFR, HER-2 and CD44 suggested the participation of this proteoglycan in early stages of tumor progression. Considering the intense proliferation of myoepithelial cells that characterizes CMT, in addition to the abundance of the mesenchymal component that could contribute to high expression of VCAN, the present study focused on differences in molecular expression of this proteoglycan and its receptors in CMT, as well as SC tumor types.

Several reports discussing the role of the extracellular matrix in tumor development have focused on ECM elements, especially versican, with regard to their versatility and function in the tumor microenvironment. These elements participate in the remodeling of the matrix and interact with neoplastic cells, triggering important signaling pathways related to loss of adhesion and the activation of proliferation, thereby favoring invasion and the development of metastasis (Lu et al., 2012; Marusyk et al., 2014; Ricciardelli et al., 2009).

Our results revealed similarities in VCAN expression between the two histological tumor types evaluated, with greater staining found in the stroma adjacent to invasive carcinomatous cells in both types, corroborating previously published findings. Relatedly, associations between VCAN overexpression and tumor malignancy and aggressivity has also been demonstrated in human breast cancer (de Wit et al., 2017; Gorter et al., 2010; Touab et al., 2002; Hosseini et al., 2018; Skandalis et al., 2011) and canine mammary tumors (Erdélyi et al., 2003).

Despite the significant differences seen in versican expression in adjacent stroma in *in situ* and invasive areas in both tumor types investigated, differential expression was less marked in SC (Fig. 1a), which is associated with a worse prognosis than CMT (Cassali et al., 2014). Comparisons between *in situ* and invasive areas of CMT and SC revealed higher versican expression in CMT in both areas. This may be explained by the proliferating myoepithelial cells capable of producing proteoglycan versican in CMTs, as previously demonstrated by Erdélyi et al. (2003) and Damasceno et al. (2014).

Damasceno et al. (2014) also showed that myoepithelial cells undergoing different stages of differentiation expresses different levels of VCAN in CMT. In early stages, myoepithelial cells adjacent to *in situ* carcinomatous areas reveal lower expression than stellate-shaped myoepithelial cells producing myxoid matrix, as well as cells adjacent to pre-condroid areas. In the present study, myoepithelium proliferation appeared to be linked to the overexpression of VCAN in CMTs compared to SC. Since little is known regarding the beneficial or harmful effects provoked by the production of this proteoglycan by this cell type, further study is needed.

Versican is responsible for several functions in the ECM, and its G3

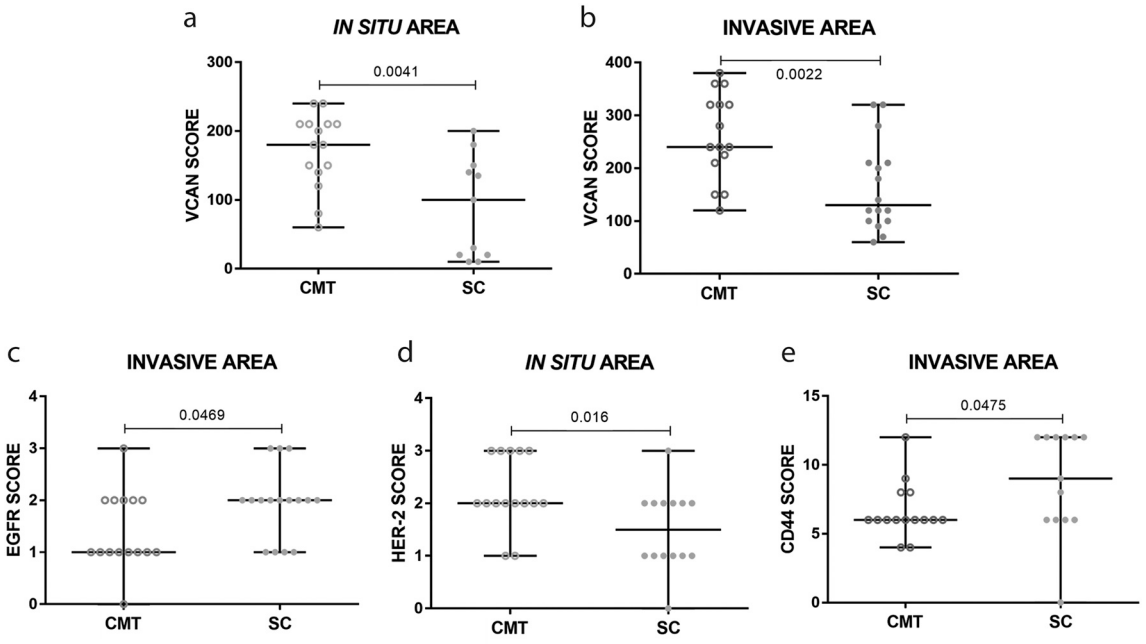

Fig. 3. Expression of VCAN, EGFR, HER-2 and CD44 in *in situ* and in invasive carcinomatous areas of CMT and SC. Mann Whitney's test, 95% confidence interval ($p < 0.05$).

Table 2
Frequencies of VCAN, EGFR, CD44 and HER-2 expression in *in situ* and invasive carcinomatous areas of SC and CMT.

	SC n/total (%)			CMT n/total (%)		
	IS	IN	*p value*	IS	IN	*p value**
VERSICAN						
(Median)	100	190	***0.0039*****	180	240	***<0.0001*****
EGFR						
0	5/17 (29.4)	1/17 (5.9)	*0.1133*	0/15 (0)	1/15 (6.7)	***0.0001*****
1	1/17 (5.9)	4/17 (23.5)		0 /15 (0)	8/15 (53.3)	
2	4/17 (23.5)	9/17 (52.9)		5/15 (33.3)	5 /15 (33.3)	
3	7/17 (41.2)	3/17 (17.6)		10/15 (66.7)	1 /15 (6.7)	
CD44						
Low (<3)	1/17 (5.9)	1/17 (5.9)	*0.9063*	1/15 (6.7)	0/15 (0)	*0.0039***
Moderate (4–6)	4/17 (23.5)	4/17 (23.5)		2 /15 (13.3)	11/15 (73.3)	
High (7–9)	3/17 (17.6)	2/17 (11.8)		8/15 (53.3)	3/15 (20)	
Very high (>9)	4/17 (23.5)	6 /17 (35.3)		4/15 (26.6)	1/15 (6.7)	
Not analyzed	5/17 (29.4)	4/17 (23.5)		0/15 (0)	0/15 (0)	
HER-2						
0	1/17 (5.9)	3/17 (17.6)	*0.1793*	0/15 (0)	1/15 (6.7)	*0.0017***
1	6/17 (35.3)	7/17 (41.2)		2/15 (13.3)	9/15 (60)	
2	6/17 (35.3)	7/17 (41.2)		8/15 (53.3)	4/15 (26.6)	
3	1/17 (5.9)	0/17 (0)		5/15 (33.3)	1/15 (6.7)	
Not analyzed	3/17 (17.6)	0/17 (0)		0/15 (0)	0/15 (0)	

Wilcoxon's test, 95% confidence interval ($p < 0.05$). (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.0001$.

domain enables interaction with receptors of epidermal growth factors (EGF), such as EGFR and HER-2 [30], in addition to promoting tumor proliferation and invasion (Ricciardelli et al., 2007; Yee et al., 2007). Here, our expression analysis of these molecules in CMT indicated higher EGFR and HER-2 expression in *in situ* carcinomatous areas compared to invasive areas. In addition, increased HER-2 expression was seen in *in situ* areas in SC. Similar patterns of expression were also described in *in situ* carcinomatous areas in canine CMT and mixed benign tumors (Bertagnolli et al., 2011; Damasceno et al., 2016a, 2016b), as well as in female breast (Aguiar et al., 2013).

The present findings demonstrate that higher expression of EGFR and HER-2 occurs in the initial phases of tumor progression, which present greater cellular activity. EGFR was found to be positively correlated with versican expression in *in situ* and invasive areas in CMTs. Damasceno et al. (2016b) observed a tendency for increased expression of both receptors in tumors with higher versican expression. This suggests that interactions between VCAN and its receptors occur at early stages of cancer, which may trigger cell proliferation events in CMT. A comparison of the expression profiles of both histological types revealed higher EGFR reactivity in invasive carcinomatous areas of SC. This finding may be supported by the fact that this tumor type is related to a worse prognosis, and exhibits different behavior than CMT. In women, aggressive carcinomas, such triple negative breast cancer, tend to express more EGFR in invasive areas than non-triple negative breast cancer (Viale et al., 2009).

Interactions between the CD44 receptor and versican, either directly or indirectly, have been associated with increased cellular motility and the development of metastasis (Heider et al., 1993; Yu and Stamenkovic,

1999). The present study observed a higher expression of CD44 in *in situ* areas compared to invasive areas in CMT. This corroborates previously published data by Damasceno et al. (2016a) in CMT and carcinosarcomas. Our comparison between invasive areas of CMT and SC revealed higher CD44 expression in SC. A study conducted by Magalhães et al. (2012) noted that CD44 is more expressed in metastatic areas of canine carcinomas, highlighting the relationship of this biomarker with tumor progression.

CD44 expression seemed to follow a similar pattern as the EGF receptors, being more expressed in *in situ* areas in CMT as well as in invasive areas in SC. Indeed, versican and CD44 expression in *in situ* areas was found to be statistically correlated in SC. It is known that these molecules have an intimate bonding relationship through hyaluronic acid linked to the globular amino terminal domain of versican (Wu et al., 2005). Yeung et al. (2013) concluded that high levels of versican expression in ovarian cancer cells in women enabled high cellular motility, as well as higher CD44 expression. These authors concluded that these cells may be undergoing adaptive processes that result in further phenotypic transformation and invasion, which may be related to epithelial-mesenchymal transition, allowing cells to gain greater motility and consequently invasive capability (Mani et al., 2008).

Our investigation of associations between versican expression and clinical-pathological factors revealed no significant relationships between the investigated clinicopathological characteristics and the expression of the proteoglycan, regardless of detection in *in situ* or invasive areas of different histological tumor types. Notably, no similar associations were reported by previous studies in humans with squamous cell carcinomas of the pharynx and lung, nor even in canine carcinomas in mixed tumors (Damasceno et al., 2012, 2016a; Pirinen et al., 2005; Pukkila et al., 2004;), despite the fact that this molecule has been implicated in tumor aggressiveness, lower survival rates and higher probability of recurrence in cases of ovarian cancer in women (Voutilainen et al., 2003).

5. Conclusions

The results of this study indicate that versican expression is associated with invasiveness in SC as well as CMT. More intense expression was seen in CMT, suggesting that the presence of myoepithelial proliferation participates in the stromal composition of this tumor subtype. Additionally, the analysis of EGFR, HER-2 and CD44 surface receptor expression revealed higher activity of these molecules mainly in earlier stages of tumor progression in CMT, but also in invasive stages of SC. Since we also observed higher expression of versican in the stroma adjacent to invasive areas, it is likely that that interactions between these molecules occur in later stages of SC. It will be important to comprehensively investigate these phenomena in order to achieve a better understanding of the relationships between these molecules in stroma adjacent to the invasion areas.

Ethics approval

All procedures in this research were approved by the Institutional Review Board for Animal Experimentation at the Gonçalo Moniz Institute of the Oswaldo Cruz Foundation (IGM-FIOCRUZ, protocol no. 009/2016).

Funding

This work was supported by a grant from Oswaldo Cruz Foundation (Fiocruz). AEL (Proc. 305938/2018-5) and GDC (Proc. 302449/2013-2) are research fellows of the National Council for Scientific and Technological Development (CNPq).

Author information

Samantha Hellen Santos Figuerêdo and Rafael da Silva Carmo Neto contributed equally to this work.

Declaration of Competing Interest

The authors declare that they have no competing interests financial or non-financial that might have influenced interpretation or presentation of the data presented in this manuscript.

Acknowledments

The authors thank oncology veterinary group of Research Center on Mammary Oncology NPqOM/HOSPMEV/UFBA and Comparative Pathology laboratory (UFMG) by support during this work.

References

Aguiar, F.N., Mendes, H.N., Bacchi, C.E., Carvalho, F.M., 2013. Comparison of nuclear grade and immunohistochemical features in situ and invasive components of ductal carcinoma of breast. Rev Bras Ginecol Obstet. 35, 97–102.

Bertagnolli, A.C., Ferreira, E., Dias, E.J., Cassali, G.D., 2011. Canine mammary mixed tumours: immunohistochemical expressions of EGFR and HER-2. Aust. Vet. J. 89, 312–317.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., Jemal, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 68, 394–424.

Cassali, G.D., Damasceno, K.A., Bertagnolli, A.C., Estrela-Lima, A., Lavalle, G.E., Di Santis, G.W., De Nardi, A.B., Fernandes, C.G., Cogliati, B., Sobral, R., et al., 2017. Consensus regarding the diagnosis, prognosis and treatment of canine mammary tumors: benign mixed tumors, carcinomas in mixed tumors and Carcinosarcomas. Brazil. J. Veterin. Pathol. 10, 87–99.

Cassali, G.D., Lavalle, G.E., De Nardi, A.B., Ferreira, E., Bertagnolli, A.C., Estrela-Lima, A., Alessi, A.C., Daleck, C.R., Salgado, B.S., Fernandes, C.G., et al., 2011. Consensus for the diagnosis, prognosis and treatment of canine mammary tumors. Brazil. J. Veterin. Pathol. 4, 153–180.

Cassali, G.D., Lavalle, G.E., Ferreira, E., Estrela-Lima, A., De Nardi, A.B., Ghever, C., Sobral, Amorim R.L., Oliveira, L.O., Sueiro, F.A.R., 2014. Consensus for the diagnosis, prognosis and treatment of canine mammary tumors. Brazil. J. Veterin. Pathol. 7, 38–69.

Damasceno, K.A., Bertagnolli, A.C., Estrela-Lima, A., Ribeiro, L.G.R., Rabelo, B.S., Campos, C.B., Barros, A.L.B., Cassali, G.D., 2012. Versican expression in canine carcinomas in benign mixed tumours: is there an association with clinical pathological factors, invasion and overall survival? BMC Vet. Res. 8, 195.

Damasceno, K.A., Bertagnolli, A.C., Estrela-Lima, A., Rabelo, B.S., Campos, L.C., Ribeiro, L.G.R., Cassali, G.D., 2014. Versican expression in myoepithelial cells from carcinomas in canine mixed mammary tumors. Vet. J. 200, 146–151.

Damasceno, K.A., Ferreira, E., Estrela-Lima, A., Gamba, C., Miranda, F.F., Alves, M.R., Rocha, R.M., de Barros, A.L., Cassali, G.D., 2016a. HER-2 and EGFR mRNA expression and its relationship with Versican in malignant matrix-producing tumors of the canine mammary gland. PLoS One 11, e0160419.

Damasceno, K.A., Ferreira, E., Estrela-Lima, A., Bosco, Y., Silva, L.P., Barros, A.L.B., Bertagnolli, A.C., Cassali, G.D., 2016b. Relationship between the expression of versican and EGFR, HER-2, HER-3 and CD44 in matrix-producing tumours in the canine mammary gland. Histol. Histopathol. 31 (6), 675–688. https://doi.org/10.14670/HH-11-705. Epub 2015 Dec 15. PMID: 26666308.

Du, W.W., Yang, W., Yee, A.J., 2013. Roles of versican in cancer biology–tumorigenesis, progression and metastasis. Histol. Histopathol. 28, 701–713.

Elston, C.W., Ellis, I.O., 1998. Assessment of histological grade. In: Systemic Pathology. The breast, 3.ed. Churchill Livingstone, London, pp. 365–384.

Erdélyi, I., Nieskens, D.H., Van Dijk, J.E., Vass, L., Nederbragt, H., 2003. Immunohistochemical evaluation of versican, in relation to chondroitin sulphate, in canine mammary tumours. Histol. Histopathol. 18, 1067–1080.

Erdélyi, I., van Asten, A.J., van Dijk, J.E., Nederbragt, H., 2005. Expression of versican in relation to chondrogenesis-related extracellular matrix components in canine mammary tumors. Histochem. Cell Biol. 124, 139–149.

Goldschmidt, M., Peña, L., Rasotto, R., Zappulli, V., 2011. Classification and grading of canine mammary tumors. Vet. Pathol. 48, 117–131.

Gorter, A., Zijlmans, H.J., van Gent, H., Trimbos, J.B., Fleuren, G.J., Jordanova, E.S., 2010. Versican expression is associated with tumor-infiltrating CD8-positive T cells and infiltration depth in cervical cancer. Modern Pathol.: Off. J. US Can. Acad. Pathol. Inc 23, 1605–1615.

Heider, K.H., Hofmann, M., Hors, E., van den Berg, F., Ponta, H., Herrlich, P., Pals, S.T., 1993. A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. J. Cell Biol. 120, 227–233.

Hernández, D., Miquel-Serra, L., Docampo, M.J., Marco-Ramell, A., Bassols, A., 2011. Role of versican V0/V1 and CD44 in the regulation of human melanoma cell behavior. Int. J. Mol. Med. 27, 269–275.

Hosseini, S.M., Jafary, F., Hosseini, M., Mahjoubi, F., 2018. VCAN gene expression and its association with tumor stage and lymph node metastasis in colorectal cancer patients. Biomed. Res. 29, 1110–1114.
Kischel, P., Waltregny, D., Dumont, B., Turtoi, A., Greffe, Y., Kirsch, S., De Pauw, E., Castronovo, V., 2010. Versican overexpression in human breast cancer lesions: known and new isoforms for stromal tumor targeting. Int. J. Cancer 126, 640–650.
Lu, P., Weaver, V.M., Werb, Z., 2012. The extracellular matrix: a dynamic niche in cancer progression. J. Cell Biol. 196, 395–406.
Magalhães, M.G., Silveira, A.C.T., Munari, D.P., Alessi, A., 2012. Behavior of CD44 receptors in mammary tumors of dogs. Open J. Veterin. Med. 2, 48–51.
Mani, S.A., Guo, W., Liao, M.J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., et al., 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133, 704–715.
Marusyk, A., Tabassum, D.P., Altrock, P.M., Almendro, V., Michor, F., Polyak, K., 2014. Non-cell-autonomous driving of tumour growth supports sub-clonal heterogeneity. Nature 514, 54–58.
Nunes, F.C., Campos, C.B., Teixeira, S.V., Bertagnolli, A.C., Lavalle, G.E., Cassali, G.D., 2018. Epidemiological, clinical and pathological evaluation of overall survival in canines with mammary neoplasms. Arquivo Brasil. Med. Veterin. Zoot. 70, 1714–1722.
Owen, L.N., 1980. WHO Classification of Tumours in Domestic Animals. World Health Organization, Geneva, pp. 3–53.
Paltian, V., Alldinger, S., Baumgärtner, W., Wohlsein, P., 2009. Expression of CD44 in canine mammary tumours. J. Comp. Pathol. 141, 237–247.
Pirinen, R., Leinonen, T., Böhm, J., Johansson, R., Ropponen, K., Kumpulainen, E., Kosma, V.M., 2005. Versican in nonsmall cell lung cancer: relation to hyaluronan, clinicopathologic factors, and prognosis. Hum. Pathol. 36, 44–50.
Pukkila, M.J., Kosunen, A.S., Virtaniemi, J.A., Kumpulainen, E.J., Johansson, R.T., Kellokoski, J.K., Nuutinen, J., Kosma, V.M., 2004. Versican expression in pharyngeal squamous cell carcinoma: an immunohistochemical study. J. Clin. Pathol. 57, 735–739.
Ricciardelli, C., Russell, D.L., Ween, M.P., Mayne, K., Suwiwat, S., Byers, S., Marshall, V. R., Tilley, W.D., Horsfall, D.J., 2007. Formation of hyaluronan- and versican-rich pericellular matrix by prostate cancer cells promotes cell motility. J. Biol. Chem. 282, 10814–10825.
Ricciardelli, C., Sakko, A.J., Ween, M.P., Russell, D.L., Horsfall, D.J., 2009. The biological role and regulation of versican levels in cancer. Cancer Metastasis Rev. 28, 233–245.
Rutnam, Z.J., Wight, T.N., Yang, B.B., 2013. miRNAs regulate expression and function of extracellular matrix molecules. Matrix Biol.: J. Int. Soc. Matrix Biol. 32, 74–85.
Skandalis, S.S., Labropoulou, V.T., Ravazoula, P., Likaki-Karatza, E., Dobra, K., Kalofonos, H.P., Karamanos, N.K., Theocharis, A.D., 2011. Versican but not decorin accumulation is related to malignancy in mammographically detected high density and malignant-appearing microcalcifications in non-palpable breast carcinomas. BMC Cancer 11, 314.
Touab, M., Villena, J., Barranco, C., Arumí-Uría, M., Bassols, A., 2002. Versican is differentially expressed in human melanoma and may play a role in tumor development. Am. J. Pathol. 160, 549–557.
Uva, P., Aurisicchio, L., Watters, J., Loboda, A., Kulkarni, A., Castle, J., Palombo, F., Viti, V., Mesiti, G., Zappulli, V., Marconato, L., Abramo, F., Ciliberto, G., Lahm, A., La Monica, N., de Rinaldis, E., 2009. Comparative expression pathway analysis of human and canine mammary tumors. BMC Genomics 10, 135.
Viale, G., Rotmensz, N., Maisonneuve, P., Bottiglieri, L., Montagna, E., Luini, A., Veronesi, P., Intra, M., Torrisi, R., Cardillo, A., Campagnoli, E., Goldhirsch, A., Colleoni, M., 2009. Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity. Breast Cancer Res. Treat. 116, 317–328.
Voutilainen, K., Anttila, M., Sillanpää, S., Tammi, R., Tammi, M., Saarikoski, S., Kosma, V.M., 2003. Versican in epithelial ovarian cancer: relation to hyaluronan, clinicopathologic factors and prognosis. Int. J. Cancer 107, 359–364.
de Wit, M., Carvalho, B., Delis-van Diemen, P.M., van Alphen, C., Beliën, J., Meijer, G.A., Fijneman, R., 2017. Lumican and versican protein expression are associated with colorectal adenoma-to-carcinoma progression. PLoS One 12, e0174768.
Wolff, A.C., Hammond, M.E., Hicks, D.G., Dowsett, M., McShane, L.M., Allison, K.H., Allred, D.C., Bartlett, J.M., Bilous, M., Fitzgibbons, et al., 2013. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 31, 3997–4013.
Wu, Y.J., La Pierre, D.P., Wu, J., Yee, A.J., Yang, B.B., 2005. The interaction of versican with its binding partners. Cell Res. 15, 483–494.
Yee, A.J., Akens, M., Yang, B.L., Finkelstein, J., Zheng, P.S., Deng, Z., Yang, B., 2007. The effect of versican G3 domain on local breast cancer invasiveness and bony metastasis. Breast Cancer Res.: BCR 9, R47.
Yeung, T.L., Leung, C.S., Wong, K.K., Samimi, G., Thompson, M.S., Liu, J., Zaid, T.M., Ghosh, S., Birrer, M.J., Mok, S.C., 2013. TGF-β modulates ovarian cancer invasion by upregulating CAF-derived versican in the tumor microenvironment. Cancer Res. 73, 5016–5028.
Yu, Q., Stamenkovic, I., 1999. Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev. 13, 35–48.