RELATIONSHIP BETWEEN THE EXPRESSION OF VERSICAN AND EGFR, HER-2, HER-3 AND CD44 IN MATRIX-PRODUCING TUMOURS IN THE CANINE MAMMARY GLAND

DAMASCENO, K.A.[1,2]; FERREIRA, E.[1]; ESTRELA-LIMA, A.[3]; BOSCO, Y.[1]; SILVA, L.P.[3]; BARROS, A.L.B.[4]; BERTAGNOLLI, A.C.[5]; CASSALI, G.D.[1*]

[1] Department of General Pathology, Biological Sciences Institute, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[2] Laboratory of Experimental Pathology, Gonçalo Moniz Research Center, Fundação Oswaldo Cruz, Salvador, BA, Brazil
[3] Department of Pathology and Clinics, School of Veterinary Medicine and Zootechny, Universidade Federal da Bahia, Salvador, BA, Brazil
[4] Department of Clinical and Toxicological Analyses, School of Pharmacy, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[5] Fepagro Animal Health, Instituto de Pesquisa Veterinária Desidério Finamor (IPVDF), Eldorado do Sul, RS, Brazil

* Corresponding author.
Department of General Pathology, Biological Sciences Institute, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
Tel.: +55 31 3409 2891.
E-mail address: cassalig@icb.ufmg.br (G.D. Cassali)

Short running title: *Versican, EGFR, HER-2, HER-3 and CD44 in canine mammary tumours*

Keywords: *versican, epidermal growth factor receptors, CD44, mammary gland tumours, canine.*

Abstract

Versican is an extracellular matrix proteoglycan that has been identified as a modulator of adhesion loss, cell motility, and tumour progression. This motility results from the interaction between versican and cell surface receptors. Studies have also demonstrated the relationship between this molecule and invasion in canine mammary tumours. Given the evidence for the participation of proteoglycans in tumour progression, this study aimed to assess versican expression and its association with cell surface receptors; human epidermal growth factor receptors 1, 2, and 3 (EGFR, HER-2, and HER-3) and CD44 through an immunohistochemical analysis of benign mixed tumours (BMTs), carcinomas in mixed tumours (CMTs), and carcinosarcomas (CSs) of the canine mammary gland. Malignant tumours were divided into low and high groups with respect to versican stromal expression. The results indicated that the BMTs showed weak stromal versican expression and correlations between the expression of stromal versican and EGFR in the epithelial membrane in benign areas ($p=0.013$, $r=0.571$). A higher stromal versican expression was observed adjacent to invasive epithelial areas compared with *in situ* areas in CMTs and CSs, suggesting a direct relationship between versican expression and invasiveness. Furthermore, the CSs exhibited a higher expression of HER-2, cytoplasmic HER-3, and CD44 in epithelial invasive cells in cases of higher stromal versican expression. Therefore, the cell surface receptors (HER-2, HER-3, and CD44) are more evident in CSs that overexpress versican in stroma adjacent to the invasive areas. These findings suggest that the association between these molecules may be directly related to the biological behaviour and invasiveness of these canine mammary tumours.

INTRODUCTION

The extracellular matrix plays a key role in creating a microenvironment favourable to neoplastic cell migration and invasion during the process of tumour development (Liotta and Kohn, 2001; Kischel et al., 2010; Canavese et al., 2011). Versican stands out among the multiple components of the neoplastic extracellular matrix because of its involvement in progression, adhesion loss, cell proliferation, and invasion (Pukkila et al., 2004; Suwiwat et al., 2004; Ricciardelli et al., 2007; Yee et al., 2007; Kischel et al., 2010; Canavese et al., 2011; Damasceno et al., 2012; Du et al., 2013).

Versican is a large hyaluronan-binding proteoglycan versican (also known as PG-M) present in the connective tissues of various organs in human adult tissues, including smooth muscle, cartilage, heart, blood vessels, skin, and the central nervous system (Bode Lesniewska et al., 1996; Wight, 2002; Choocheep et al., 2010). The structure of the versican protein consists of a glycosaminoglycan (GAG) attachment domain of variable size, surrounded by two external globular domains, G1 and G3 (Kischel et al., 2010). The amino-terminal globular end (G1) binds to the glycosaminoglycan (GAG) hyaluronan, and the carboxy-terminal globular domain (G3) resembles the selectin family of proteins, consisting of a C-type lectin adjacent to two epidermal growth factor (EGF) domains and a complement regulatory region (Bode Lesniewska et al., 1996; Wight, 2002; Kischel et al., 2010). The alternate splicing of two exons, namely GAGa (encoded by exon 7) and GAGb (encoded by exon 8), generates three variants, V1, V2 and V3. A recent study identified a new isoform, called V4 (Kischel et al., 2010).

In the extracellular matrix, versican binds to various molecules, including tenascin, fibulin-1, fibrillin, fibronectin, CD44, L-selectin, and integrin β1 (Bode Lesniewska et al., 1996; Aspberg et al., 1999; Wu et al., 2004; Wu et al., 2005). These interactions with elements of the extracellular matrix ensure tissue integrity and regulate cell proliferation and differentiation (Sheng et al., 2006).

The migration of neoplastic cells arises from the interaction between versican and cell surface receptors of neoplastic cells, including the cluster of differentiation 44 (CD44) and epidermal growth factor receptor (EGFR), resulting in the formation of a complex capable of triggering intracellular signalling pathways that regulate the proliferation and migration of neoplastic cells in the extracellular matrix (Hernandez et al., 2011; Ween et al., 2011).

The existence of a EGFR/HER2 receptor complex able to interact with CD44 was recognized in MeWo melanoma cells in which the V3 isoform maintains the ability to bind EGF receptors (EGFR) through the EGF-like subdomains of G3 (Hernandez et al., 2011). The mechanisms that alter the expression of this proteoglycan are still poorly understood, although its role in modulating adhesion loss and cell motility has also been identified in cases of breast cancer metastasis (Suwiwat et al., 2004; Yee et al., 2007).

In veterinary medicine, increased versican expression was observed in areas of carcinomatous invasion in canine colorectal adenomas and adenocarcinomas and in stroma adjacent to invasive areas of the canine mammary carcinomas, indicating that alterations in these proteoglycan levels were associated with the tumour progression and invasion process (Mukaratirwa and Nederbragt, 2002; Erdélyi et al., 2003; Damasceno et al., 2012).

Matrix-producing mammary tumours, including benign mixed tumours (BMTs) and carcinomas in mixed tumours (CMTs), are common in small animal clinical practice (Cassali et al., 2014). These tumours have been proposed as spontaneous models of malignant transformation (Cassali et al., 2012). BMTs are characterised by benign epithelial, myoepithelial, and mesenchymal proliferation. In CMTs, the epithelial component undergoes malignant transformation (Misdorp, 2002). Another histological type characterised by myxoid, chondroid, and osteoid matrix production is the carcinosarcoma (CS); however, both the epithelial and mesenchymal components are malignant. CS is less common in bitches and has a poor prognosis (Cassali et al., 2014).

Given that proteoglycan versican participates in tumour progression, the aim of this study was to assess versican expression and its relationship with clinicopathological factors (involvement of lymph nodes, presence of distant metastasis, stage, and histological grade) and the cell surface receptor EGFR, human epidermal growth factor receptors 2 and 3 (HER-2, HER-3), and CD44 of matrix-producing tumours of the canine mammary gland.

MATERIALS AND METHODS

Selection of cases

In this study, 18, 41, and 18 samples of BMTs, CMTs, and CSs, respectively, were selected at the Comparative Pathology Laboratory (*Universidade Federal de Minas Gerais*) and Veterinary Pathology Laboratory (*Universidade Federal da Bahia*). Samples of

mammary tumours were obtained from female dogs of any breed or age that had undergone mastectomy.

Anatomopathological study

Clinical staging was conducted based on tumour size (T), neoplastic involvement of regional lymph nodes (N), and presence of distant metastases (M) according to the Tumour-Node-Metastasis (TNM) staging system established by the World Health Organization (WHO) for canine mammary tumours (modified from Owen, 1980). These data were collected from the clinical, radiological, and pathological records of each animal.

Four-micrometre histological sections were prepared from selected blocks and stained using the hematoxylin-eosin method. The histological type was confirmed according to the standards proposed by Misdorp et al., (1999) and the Consensus for the Diagnosis, Prognosis, and Treatment of Canine Mammary Tumours (Cassali et al., 2014). Malignant invasive epithelial components in CMTs and CSs were graded according to the Nottingham System (Elston and Ellis, 1998), which included tubule formation, nuclear pleomorphism, and mitotic index.

The *in situ* areas were defined through the observation of epithelial cells in a tubular arrangement with myoepithelial cell layer and basal membrane integrity shown by HE (Cassali et al., 2014).

Immunohistochemistry

The primary antibodies used in the immunohistochemical analysis were versican (1:50, clone 12C5, DSHB, Iowa, USA), EGFR (1:100, clone 31G7, Invitrogen, California, USA), HER-2 (1:200, polyclonal, Dako, Glostrup, Denmark), HER-3 (1:100, polyclonal, GeneTex, California, USA), and CD44 (1:200, clone IM7, Santa Cruz, Texas, USA).

For this technique, 3-µm sections were cut from one representative block of each case and collected on gelatinised slides. Tissue sections were deparaffinised, rehydrated in a graded ethanol series, and subjected to heat-induced antigen retrieval (water bath at 98°C for 20 minutes) with a target retrieval solution (DAKO) at pH 6.0, except for the slides intended for versican and EGFR staining. For versican, enzymatic recovery was performed using 0.5 U/ml chondroitinase ABC (*Proteus vulgaris*; Sigma Chemicals) in 0.25 M Tris

buffer (pH 8.0) with 0.18 M sodium chloride and 0.05% bovine serum albumin (BSA) at a temperature of 37°C for 1 hour and 30 minutes. A 0.25 M Tris buffer solution (pH 8.0) with 0.1 M 6-amino-n-caproic acid and 5 mM benzamidine hydrochloride was used for 30 minutes to inhibit the protease activity (adapted from Erdélyi et al., 2005). Enzymatic recovery of EGFR was performed using HCl-diluted pepsin at 37°C for 30 minutes. All of the slides were incubated for 15 minutes in 3% hydrogen peroxide in methanol to block the endogenous peroxidase activity. Subsequently, the slides were covered with 10% normal rabbit serum in phosphate buffered saline (PBS) for 10 minutes and then incubated with the primary antibody for 1 hour at 37°C (CD44) or overnight at 4°C (versican, EGFR, HER-2 and HER-3). Subsequently, the method of polymerization was applied, with the identification based on secondary antibodies (ADVANCE HRP - ready to use - DakoCytomation). Diaminobenzidine was used as the chromogen, and the sections were counterstained with Mayer's hematoxylin, hydrated, and mounted in synthetic medium.

Negative controls were prepared by omitting the primary antibody. Canine mammary tumours previously known to express HER-2 and HER-3 and tissue with abundant myxoid matrix expressing versican were used as positive controls. Skin and lymph nodes were used as positive controls for EGFR and CD44, respectively.

Immunohistochemical evaluation

Versican expression in the stroma of adjacent areas to normal mammary gland and benign, malignant *in situ* and invasive epithelial proliferation was assessed by the semiquantitative scoring system proposed by Skandalis et al. (2011), which includes (i) the overall percentage of positively stained tissue (0-100%) and (ii) staining intensity for proteoglycan using the following scales: (1) negative or very weak staining, (2) weak positive staining, (3) moderately positive staining, and (4) strongly positive staining. The versican expression level was then calculated by the product of the percentage (i; 0-100%) of tissue section positive staining and the intensity of this staining (ii; 1-4). Based on the final results of this evaluation, a median versican expression score was obtained for the CMT (median=280) and CS (median=180) invasive areas. Thus, two distinct groups were determined on each histological type: group 1 was represented by cases with values below the median and was considered to have low versican expression; group 2 was represented

by cases with values equal and above the median and was considered to have versican overexpression (Damasceno et al., 2012).

EGFR and HER-2 expression was assessed in epithelial cells using the scores defined according to the consensus of the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP; Wolff et al., 2013) and adapted from Bertagnolli et al. (2011) as follows: (-), no staining; (1+), weak incomplete membrane staining in more than 10% of tumour cells; (2+), incomplete and/or weak-to-moderate membrane staining in more than 10% of tumour cells or complete and strong staining in less than 10% of tumour cells; and (3+), complete and strong staining in at least 10% tumour cells.

HER-3 expression was assessed in the membrane, cytoplasm, and nucleus of the epithelial cells and was divided into four groups: high expression (3+, >35%), moderate expression (2+, 18–35%), low expression (1+, 1–17%), and no expression (0, 0%;) (adapted from Kim et al., 2011).

The intensity of CD44 immunoreactivity in the epithelial cells was assessed as follows: (0) no staining; (1) low staining; (2) moderate staining; and (3) strong staining. The number of stained cells was estimated as follows: (0) no staining; (1) 1-25% cells stained; (2) 26-50% cells stained; (3) 51-75% cells stained; and (4) >75% cells stained. The immunohistochemical score was calculated by multiplying the intensity and number of stained cells. A score of ≤3 was regarded as ‘‘low’’, a score of 4-6 as ‘‘moderate’’, a score of 7-9 as ‘‘high’’, and a score of ≥10 as ‘‘very high’’ (Paltian et al., 2009).

Only epithelial component and adjacent stroma were evaluated in this study. At least five fields of each of the normal epithelial cells, epithelial benign, *in situ*, and invasive carcinomatous proliferation in BMTs, CMTs, and CSs were analysed in this study.

Statistical analysis

Statistical analyses were performed using the Graph Pad Prism v.5 software (San Diego, CA). The Kruskal Wallis test was used to assess whether there were differences in the animal's age (mean), tumour size, and stage. The Mann–Whitney U test was used to assess whether there were differences in lymph node involvement, presence of distant metastasis, and histological grade only in CMTs and CSs. The difference in versican, EGFR, HER-2, HER-3, and CD44 expression between *in situ* and invasive areas was

assessed using the Wilcoxon signed-rank test, and a Mann–Whitney U test (nonparametric data) was used to assess whether the receptor expression differed between groups (with high and low versican expression). The data were also subjected to Spearman's rank correlation coefficients. The values were considered significant when $p<0.05$.

Ethical aspects

All procedures were performed under the guidelines and with the approval of the Ethics Committee in Animal Experimentation (CETEA/UFMG), protocol 0053/11.

RESULTS

Clinicopathological factors

Female dogs diagnosed with BMT, CMT, and CS had a mean age of 8.7 years (ranging from 4 to 12 years), 10.2 years (ranging from 4.5 to 16 years), and 11.9 years (ranging from 8 to 16 years), respectively. The mean age among the histological types showed a significant difference ($p=0.0036$).

The mean tumour diameter of the BMTs, CMTs, and CSs was 1.6 cm (0.2-6 cm), 4 cm (0.5-12 cm), and 7.2 cm (3.5-14 cm), respectively, with a significant difference among groups ($p=0.0001$).

Other significant differences were observed between the neoplastic types studied in relation to the occurrence of lymphatic and systemic metastasis, as well as histological grade. Pulmonary metastasis occurred in eight cases.

The clinicopathological characteristics of the histological types are listed in Table 1. The presence of lymph node metastasis only showed a direct relationship with higher versican expression in the stroma adjacent to *in situ* areas in CMTs ($p=0.046$, $r=0.274$).

Versican, EGFR, HER-2, HER-3 and CD44 expression in BMTs, CMTs, and CSs

BMTs

Absent or weak versican expression was observed in the adjacent normal mammary gland stroma. Low EGFR and HER-2 immunoreactivity was detected in membrane epithelial cells in 72.7% and 53.8% of cases, respectively. Usually, these cells showed

weak cytoplasmic (68.8% of cases), no nuclear and membrane HER-3 expression (56.3% and 93.8% of cases, respectively) and weak membrane CD44 expression (61.6% of cases).

In areas of benign epithelial cell proliferation, the versican staining was weak in the adjacent stroma (Fig. 1b). The epithelial cells revealed weak and moderate membrane EGFR expression (56.3% and 43.7%, respectively) (Fig. 1c) and moderate and incomplete membrane HER-2 expression (62.5%) (Fig. 1d). Cytoplasmic HER-3 expression was usually mild or moderate (56.3% and 37.4%, respectively) (Fig. 1e). Nuclear HER-3 staining was absent or weakly observed (both representing 43.8% of the cases), and membrane HER-3 expression was absent in most cases (87.5%). Commonly, benign epithelial cells revealed moderate CD44 expression (88.2%) (Fig. 1f).

Versican immunoreactivity in the adjacent stroma to normal epithelial areas was weak and even absent compared with that of areas adjacent to benign epithelial proliferation (p=0.0021), which were characterised by moderate expression (Fig. 1b). Lower EGFR (p=0.0034) expression was observed in normal gland epithelial cells compared to epithelial cells with benign proliferation. A positive correlation was observed between versican and EGFR in benign areas (p=0.013, r= 0.571). HER-2 expression did not differ between normal and benign epithelial areas. Higher cytoplasmic (p=0.0021) and higher nuclear (p=0.0263) HER-3 expression was observed in normal areas, compared with membrane expression. Benign epithelial cells showed higher cytoplasmic staining than nuclear (p=0.0209) or membrane (p=0.0012) HER-3 staining. Nuclear HER-3 expression was also more significant than membrane expression in these areas (p=0.0330). CD44 (p=0.0023) expression was weaker in normal gland epithelial cells than in benign epithelial proliferation. Results are summarized in Table 2 and the statistically significant differences are presented in Fig. 2 and 4.

CMTs

The adjacent stroma in *in situ* carcinomatous areas, in most of the cases, showed moderate versican expression (Fig. 3b). *In situ* carcinomatous cells commonly displayed strong membrane EGFR expression (63.2%) (Fig. 3d) and moderate membrane HER-2 expression (59.5%). Weak HER-3 staining was observed in the cytoplasm of these carcinomatous cells (71.4%), and expression of nuclear and membrane HER-3 was absent

(68.6% and 88.6%, respectively) in most cases. Strong membrane staining was usually observed for CD44 (42.1%) (Fig. 3h).

In invasive carcinomatous areas, strong versican expression was evidenced in the adjacent stroma (Fig. 3c). The invasive malignant epithelial cells usually exhibited weak or moderate membrane EGFR immunoreactivity (both representing 42.1% of the cases) (Fig. 3e) and weak membrane HER-2 expression (51.3%) (Fig. 3f). Commonly, these cells revealed weak cytoplasmic HER-3 staining (64.7%) (Fig. 3g) and absence of membrane and nuclear reactivity (82.4% and 91.2%, respectively). Moderate membrane CD44 expression was observed in most of the cases (56.5%) (Fig. 3j).

The *in situ* and invasive carcinomatous areas of CMTs were analysed individually, and a higher versican expression was noted in the adjacent stroma to invasive epithelial areas ($p<0.0001$). Lower EGFR ($p<0.0001$), HER-2 ($p<0.0001$) and CD44 ($p=0.0033$) expression was observed in invasive carcinomatous cells than in *in situ* areas. HER-3 staining was more significant in the cytoplasm than in the nucleus ($p<0.0001$) and membrane ($p<0.0001$) of the epithelial cells in the *in situ* and invasive areas. Results are summarized in Table 3 and the statistically significant differences are presented in Fig. 2 and 4.

CSs

Fourteen CSs exhibited *in situ* carcinomatous areas, in which moderate versican expression was observed in the adjacent stroma (Fig. 5b). In the *in situ* areas, most carcinomatous cells showed strong membrane EGFR expression and moderate membrane HER-2 expression (58.3% and 81.8% of cases, respectively) (Fig. 5e). Usually, the *in situ* carcinomatous cells presented a weak cytoplasmic HER-3 reactivity (71.4%), and absence of nuclear and membrane HER-3 expression (78.6% and 100%, respectively). Moderate membrane CD44 expression was frequently observed (60%) (Fig. 5h).

In invasive carcinomatous areas, moderate-to-strong versican expression was observed in the adjacent stroma. Most invasive carcinomatous cells displayed a moderate membrane EGFR expression (62.4%) (Fig. 5d) and a low membrane HER-2 expression (55.6%) (Fig. 5f). These cells commonly presented weak cytoplasmic immunostaining (70.6%) (Fig. 5g) and no nuclear and membrane reactivity (88.2% and 100%, respectively)

for HER-3. Moderate membrane CD44 expression was shown in the majority of cases (57.2%) (Fig. 5i).

A higher expression of versican (p=0.039) was observed in the stroma adjacent to invasive carcinomatous areas, and a lower expression of EGFR (p=0.0297) and HER-2 (p=0.0369) was observed in invasive carcinomatous cells, compared with the *in situ* areas (Fig. 5). Correlations were observed between the expression of versican and CD44 (p=0.011, r=0.710) in the *in situ* carcinomatous areas and between versican and HER-2 (p=0.047, r=0.406) and CD44 (p=0.002, r=0.665) in the invasive carcinomatous areas. Results are summarized in Table 2 and the statistically significant differences are presented in Fig. 2 and 4.

Versican high and low expression groups in CTM and CS: association with clinicopathological parametres and the immunohistochemical markers EGFR, HER-2, HER-3, and CD44

The clinicopathological characteristics of the versican groups are shown in Table 3.

In CMTs (280) and CSs (180), the medians that defined the cut-off dividing high and low versican expression were determined based on versican expression analysis in the stroma adjacent to invasive areas. Group 1 (low versican expression) and group 2 (high versican expression) were defined within each histological type.

In CMTs, neoplasms with low versican expression showed greater HER-2 immunostaining in the *in situ* areas (p=0.0136), while the high versican expression group displayed greater cytoplasmic HER-3 staining in the invasive areas (p=0.0415; Table 4).

In CSs, the high versican expression group exhibited higher HER-2 expression in invasive areas (p=0.0375), cytoplasmic HER-3 in invasive (p=0.0340) areas, and CD44 expression in invasive (p=0.0474) areas (Table 4).

DISCUSSION

Neoplastic cells can induce modifications in the tumour stroma through the production and secretion of cytokines and growth factors (Liotta and Kohn, 2001). Versican, a proteoglycan that forms the extracellular matrix, has been highlighted because overexpression of this molecule is strongly associated with tumour invasion, migration, and

progression and therefore yields worse prognoses in different types of cancer (Ricciardelli et al., 1998; Zhang et al., 1998; Ricciardelli et al., 2002; Pukkila et al., 2004, 2007; Kodama et al., 2007; Sheng et al., 2007). In canine mammary tumours, authors have reported the accumulation of versican in both the stroma surrounding the tumour cells and the matrix produced by the proliferating myoepithelial cells in mixed tumours (Hinrichs et al., 1999; Erdelyi et al., 2003, 2005; Damasceno et al., 2014).

This study assessed the possible association between versican expression and clinicopathological factors in the different tumour types. A positive correlation between versican expression in *in situ* areas and lymph node metastasis was observed in CMT. Suwiwat et al. (2004) observed that versican accumulation in peritumoural areas may be considered a strong negative predictor of recurrence in women with invasive breast carcinomas with negative lymph node status. In human lung carcinoma, correlations between the expression of this proteoglycan and tumour type and histological grade were observed by Pirinen and collaborators (2005). Theses authors showed correlations between versican and lymph node status, tumour recurrence, and staging only in adenocarcinomas (Pirinen et al., 2005). No other correlations were observed between clinicopathological factors and versican expression in the present study.

The accumulation of the proteoglycan versican in peritumoural stroma may be a possible factor supporting tumour growth and metastasis of breast cancers in women (Kischel et al., 2010; Skandalis et al. 2011). The stromal versican expression in BMTs was more discreet in areas adjacent to the normal breast compared with that in benign epithelial proliferations, which is similar to the findings reported by Erdeliy et al. (2003). Malignant tumours, represented herein by CMT and CS, showed weak to moderate versican expression in the stroma surrounding the *in situ* carcinomatous areas and overexpression of this molecule in invasive areas. This result confirms previous analyses in epithelial tumours of the canine mammary gland (Erdeliy et al., 2003; Damasceno et al., 2012) and breast and prostate cancer in humans, wherein the relationship between versican overexpression and the infiltrative capacity acquired by neoplastic cells was observed (Suwiwat et al., 2004; Ricciardelli et al., 2007; Yee et al., 2007; Skandalis et al., 2011).

The mechanism that regulates the signalling pathways induced by the interaction between versican and neoplastic cells remains poorly understood (Zhang et al., 1998; Sheng

et al., 2007). One of the studied pathways is mediated by the interaction between versican and cell surface receptors, including EGF receptors and CD44 (Kawashima et al., 2000; Wu et al., 2005).

EGFR expression showed a positive correlation with versican expression in benign areas of BMTs in this study. These findings indicate a possible interaction between the molecules in the stages of benign epithelial proliferation. However, the interaction between the G3 domain of this proteoglycan and EGFR in experimental models (Du et al., 2010) is apparently important for local invasiveness and metastasis because it contributes to the survival, dissemination, and angiogenesis of neoplastic cells (Yee et al., 2007). In addition, increased G3 in murine mammary tumour cells has been shown to increase tumour growth, migration, proliferation, and metastasis through EGFR up-regulation (Du et al., 2010). In this paper, no significant association between EGFR and versican in invasive carcinomatous areas was observed.

Malignant tumours assessed in the present study showed higher HER-2 expression when proteoglycan versican was overexpressed, indicating an association between molecules in these tumours. These results differ from the findings by Skandalis et al. (2011), who found no correlation between the immunohistochemical expressions of both proteins assessed in mammary carcinomas in women. Studies indicating the relationship between HER-2 and versican expression are scarce.

Overexpression of the EGF receptor family by tumour cells has been associated with aggressiveness and, consequently, a poor prognosis (Reis Filho et al., 2005; Yang et al., 2011). Researchers have suggested that molecular changes involved in the progression from *in situ* to invasive areas, including changes in EGFR and HER-2 expression, occur even before the morphologic manifestation of invasiveness (Aguiar et al., 2013). In canine mammary tumours, Bertagnolli et al. (2011) reported that the expression of EGFR and HER-2 showed no significant difference between the *in situ* and invasive epithelial components in CMT. However, the present study observed a higher membrane expression of EGFR and HER-2 in the *in situ* areas than in invasive areas. The expression of these molecules in non-invasive areas suggests their involvement in the early stages of tumour progression (Bertagnolli et al., 2011). Morphological heterogeneity, a typical characteristic of the studied tumours, also contributes to the discrepancy in results found in the literature.

The role of HER-3 in tumour progression and its prognostic definition is poorly understood. According to the literature, this molecule may be related to aggressiveness in malignant breast tumours in women and female dogs (Bieche et al., 2003; Witton et al., 2003; Kim et al., 2011). Chiu et al. (2010) observed that HER-3 overexpression is an important prognostic indicator for women diagnosed with invasive breast cancer. In a study of experimental models of mammary tumours, Vaught et al. (2012) showed that HER-3 was expressed in preneoplastic HER-2 overexpressing mammary epithelial lesions, indicating that this relationship is necessary for cell growth and suggesting the involvement of these molecules in the malignant progression of breast lesions. In the present study, no difference in HER-3 expression was observed between normal epithelial and benign areas or between *in situ* and invasive carcinomatous areas in the studied histological types. However, cytoplasmic HER-3 staining was more significant in invasive carcinomatous areas in the cases of CMT and CS with versican overexpression, indicating a relationship between the proteoglycan and HER-3.

CD44 interacts directly or indirectly with versican (Hernández et al., 2011). In this study, the overexpression of this molecule was significant in invasive carcinomatous areas in CS as well as overexpression of versican, suggesting a relationship between these molecules. According to the literature, CD44 overexpression has been associated with a poor prognosis in breast cancer (Buess et al., 2009). The interaction between CD44 and other cell surface receptors was analysed in a study on melanomas, which showed that this protein controls the proliferation and migration of neoplastic cells through binding of the V3 isoform of versican to CD44 and EGFR, which in turn triggers a signalling pathway capable of altering the growth and migration of those cells in the stroma (Hernández et al., 2011). Other researchers have suggested an association between higher expressions of hyaluronic acid, CD44, and versican in the peritumoural stroma in ovarian carcinomas in women (Ween et al., 2011). Therefore, the results shown in this study suggest that a higher expression of those molecules and a greater association between receptors/proteoglycan are associated with the tumour progression of CMTs and CSs.

The present findings indicate the involvement of versican in the cell invasion processes of CMTs and CSs in the canine mammary gland. Cell surface receptors (HER-2, HER-3 and CD44) display a higher expression in more aggressive tumours (CS) that

overexpress versican in the adjacent stroma to invasive areas in these tumours. These data suggest that the interaction between those molecules may be directly associated with tumour aggressiveness, although the tumour progression mechanisms triggered by this interaction in matrix-producing tumours must be elucidated.

ACKNOWLEDGEMENTS

This research was supported by *Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), *Conselho Nacional de Desenvolvimento e Pesquisa* (CNPq), *Fundação de amparo à pesquisa do Estado de Minas Gerais* (FAPEMIG), and *Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais*.

REFERENCES

Aguiar F.N., Mendes H.N., Bacchi C.E. and 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.

Aspberg A., Adam S., Kostka G., Timpl R. and Heinegård D. (1999). Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. J. Biol. Chem. 274, 20444-20449.

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

Bieche I., Onody P., Tozlu S., Driouch K., Vidaud M. and Lidereau R. (2003). Prognostic value of ERBB family mRNA expression in breast carcinomas. Int. J. Cancer 106, 758-765.

Bode-Lesniewska B., Dours-Zimmermann M.T., Oderma'it B.E., Briner J., Heitz P.U. and Zimmermann D.R. (1996). Distribution of the large aggregating proteoglycan versican in adult human tissues. J. Histochem. Cytochem. 44, 303-312.

Buess M., Rajski M, Vogel-Durrer BM, Herrmann R and Rochlitz C. (2009). Tumor-endothelial interaction links the CD44(+)/CD24(−) phenotype with poor prognosis in early-stage breast cancer. Neoplasia 11, 987-1002.

Canavese G., Candelaresi G., Castellano I. and Mano M.P. (2011). Expression of proteoglycan versican in *in situ* breast lesions: Relations between stromal changes, histotype, and invasion. Pathol. Res. Pract. 207, 97-103.

Cassali G.D., Bertagnolli A.C., Ferreira E., Damasceno K.A., Gamba C.O. and Campos C.B. (2012). Canine Mammary Mixed Tumours: A Review. Vet. Med. Int. 2012, 1-7.

Cassali G.D., Lavalle G.E., Ferreira E., Estrela-Lima A., Nardi A.B., Fernandes C.G., Sobral R. A., Amorim R.L., Oliveira L.O., Sueiro F.A. R., Beserra H.E.O., Bertagnolli A.C., Gamba C.O., Damasceno K.A., Campos C.B., Araujo M.R., Campos L.C., Monteiro L.N., Nunes F. C., Horta R.S., Reis D.C., Luvizzoto M.C.R., Magalhães G.M., Raposo J.B., Ferreira A.M.R., Tanaka N.M., Grandi F., Ubukata R., Batschinski K., Terra E.M., Salvador R.C.L., Jark P.C., Delecrodi J. E.R., Nascimento N.A., Silva D.N., Silva L.P., Ferreira K.C.R.S., Frehse M.S., Santis G.W., Silva E.O., Guim T.N., Kerr B., Cintra P.P., Silva F.B.F., Leite J.S., Mello M.F.V., Ferreira M.L.G., Fukumasu H., Salgado B.S. and Torres R. (2014). Consensus for the Diagnosis, Prognosis and Treatment of Canine Mammary Tumors – 2013. Braz. J. Vet. Pathol. 7, 38-69.

Chiu C.G., Masoudi H., Leung S., Voduc D.K., Gilks B., Huntsman D.G. and Wiseman. S.M. (2010). HER-3 Overexpression Is Prognostic of Reduced Breast Cancer Survival: A Study of 4046 Patients. Ann. Surg. 251, 1107-1116.

Choocheep, K., Hatano S., Takagi H., Watanabe H., Kimata K., Kongtawelert P., Watanabe H. (2010). Versican facilitates chondrocyte differentiation and regulates joint morphogenesis. J. Biol. Chem. 285, 21114-21125.

Damasceno K.A, Bertagnolli A.C., Estrela-Lima A., Ribeiro L.G.R., Rabelo B.S., Campos C.B., Barros A.L.B. and 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, 1-10.

Damasceno K.A., Bertagnolli A.C., Estrela-Lima A., Rabelo B.S., Campos L.C., Ribeiro L.G.R. and Cassali G.D. (2014). Versican expression in myoepithelial cells from carcinomas in canine mixed mammary tumors. Vet. J. 200, 146-51.

Du W.W., Yang B.B., Shatseva T.A., Yang B.L., Deng Z., Shan S.W., Lee D.Y., Seth A. and Yee A.J. (2010). Versican G3 Promotes Mouse Mammary Tumor Cell Growth, Migration, and Metastasis by Influencing EGF Receptor Signaling. PLoS ONE 5, 1-14.

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

Elston C.W. and Ellis I.O. (1998). Assessment of histological grade. In: *Systemic Pathology. The breast*. 3rd ed. London. Churchill Livingstone. pp. 365-384.

Erdélyi I., Nieskens D.H.M., Dijk J.E., Vass L. and Nederbragt H. (2003). Immunohistochemical evaluation of versican, in relation to chondroitin sulphate, in canine mammary tumors. Histol. Histopathol. 18, 1067-1080.

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

Hernandez D., Miquel-Serra L., Docampo M.J., Marco-Ramell A., Cabrera J., Fabra A., and Bassols A. (2011). V3 Versican Isoform Alters the Behavior of Human Melanoma Cells by Interfering with CD44/ErbB-dependent Signaling. J. Biol. Chem. 286, 1475-1485.

Hinrichs, U., Rutteman, G.R. and Nederbragt, H. (1999). Stromal accumulation of chondroitin sulphate in mammary tumours of dogs. Br. J. Cancer, 80, 1359-1365.

Kawashima H., Hirose M., Hirose J., Nagakubo D., Plaas A.H.K. and Miyasaka M. (2000). Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. J. Biol. Chem. 275, 35448-35456.

Kim J.H., Im K.S., Kim N.H., Yhee J.Y., Nho W.G. and Sur J.H. (2011). Expression of HER-2 and nuclear localization of HER-3 protein in canine mammary tumors: Histopathological and immunohistochemical study. Vet. J., 189, 318-322.

Kischel P., Waltregny D., Dumont B., Turtoi A., Greffe Y., Kirsch S., Pauw E.D. and Castronovo V. (2010). Versican overexpression in human breast cancer lesions: known and new isoforms for stromal tumor targeting. Int. J. Cancer, 126, 640-650.

Kodama J., Hasengaowa, Kusumoto T., Seki N., Matsuo T., Ojima Y., Nakamura K., Hongo A. and Hiramatsu Y. (2007). Prognostic significance of stromal versican expression in human endometrial cancer. Ann. Oncol., 18: 269-274.

Liotta L.A and Kohn E.C. (2001). The micro environment of the tumor – host interface. *Nature* 411, p. 375-379.

Misdorp W., Else R.W., Hellmén E. and Lipscomb T.P. (1999). Histological classification of the mammary tumors of the dog and the cat. In: WHO International histological classification tumors of domestic animals. 2nd ed. Washington. DC, AFIP. pp 18-27.

Misdorp W. (2002). Tumors of the mammary gland. In: tumors in domestic animals. Meuten D.J. 4 ed. Iowa State. University of California, pp 575-606.

Mukaratirwa S. and Nederbragt H. (2002). Tenascin and proteoglycans: the role of tenascin and proteoglycans in canine tumours. Res. Vet. Sci. 73, 1-8.

Owen L.N. (1980). TNM Classification of tumors in Domestic Animals. In: World Health Organization. 1st ed. Geneva, pp 26-32.

Paltian V., Alldinger S., Baumgartner W. and Wohlsein P. (2009). Expression of CD44 in Canine Mammary Tumours. J. Comp. Pathol. 141, 237-247.

Pirinen R., Leinonen T., Bohm J., Johansson R., Ropponen K., Kumpulainen E. and Kosma V. (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.T., Virtaniemi J.A., Kumpulainen E.J., Johansson R.T., Kellokoski J.K., Nuutinen J. and Kosma V.W. (2004). Versican expression in pharyngeal squamous cell carcinoma: an immunohistochemical study. J. Clin. Pathol. 57, 735-739.

Pukkila M., Kosunen A., Ropponen K., Virtaniemi J., Kellokoski J., Kumpulainen E., Pirinen R., Nuutinen J., Johansson R. and Kosma V. (2007). High stromal versican expression predicts unfavourable outcome in oral squamous cell carcinoma. J. Clin. Pathol. 60, 267-272.

Reis-Filho J.S., Milanezi F., Carvalho S., Simpson P.T., Steele D., Savage K., Lambros M.B.K., Pereira E.M., Nesland J.M., Lakhani S.R. and Schmitt F.C. (2005). Metaplastic breast carcinomas exhibit EGFR, but not HER2, gene amplification and overexpression: immunohistochemical and chromogenic in situ hybridization analysis. Breast Cancer Res. 7, R1028-R1035.

Ricciardelli C., Mayne K., Sykes P.J., Raymond W.A., McCaul K., Marshall V.R. and Horsfall D.J. (1998). Elevated levels of versican but not decorin predict disease progression in early stage prostate cancer. Clin. Cancer Res. 4, 963-971.

Ricciardelli C., Brooks J.H., Suwiwat S., Sakko A.J., Mayne K., Raymond W.A., Seshadri R., LeBaron R.G. and Horsfall D.J. (2002). Regulation of Stromal Versican Expression by Breast Cancer Cells and Importance to Relapse-free Survival in Patients with Node-negative Primary Breast Cancer. Clin. Cancer Res. 8, 1054-1060.

Ricciardelli C., Russell D.L., Ween M.P., Mayne K., Suwiwat S., Byers S., Marshall V.R., Tilley W.D. and 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.

Sheng S., Wang G., La Pierre D.P., Wen J., Deng Z., Wong C.K.A., Lee D.Y., Yang B.B. (2006). Versican Mediates Mesenchymal–Epithelial Transition. Mol. Biol. Cell 17, 2009-2020.

Sheng W., Dong H., Lee D.Y., Lu W.Y. and Yang A.B. (2007). Versican Modulates Gap Junction Intercellular Communication. J. Cell Physiol. 211, 210-219.

Skandalis S.S., Labropoulou V.T., Ravazoula P., Likaki-Karatza E., Dobra K., Kalofonos H.P., Karamanos N.K. and 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, 1-13.

Suwiwat S., Ricciardelli C., Tammi R., Tammi M., Auvinen P., Kosma V.M., LeBaron R.G., Raymond W.A., Tilley W.D. and Horsfall D.J. (2004). Expression of Extracellular Matrix Components Versican, Chondroitin Sulfate, Tenascin, and Hyaluronan, and Their Association with Disease Outcome in Node-Negative Breast Cancer. Clin. Cancer Res. 10, 2491-2498.

Vaught D.B, Stanford J.C., Young C., Hicks D.J., Wheeler F., Rinehart C., Sanchez V., Koland J., Muller W.J., Arteaga C.L. and Cook R.S. (2012). Breast Epithelium and Tumor Formation. Cancer Res. 72, 2672-2682.

Ween M.P., Oehler M.K. and Ricciardelli C. (2011). Role of Versican, Hyaluronan and CD44 in Ovarian Cancer Metastasis. Int. J. Mol. Sci. 12, 1009-1029.

Wight, T. N. (2002). Versican: a versatile extracellular matrix proteoglycan in cell biology. Curr. Opin. Cell Biol. 14, 617-623.

Witton C.J., Reeves J.R., Going J.J., Cooke T.G. and Bartlett J.M. (2003). Expression of the HER1–4 family of receptor tyrosine kinases in breast cancer. J. Pathol. 200, 290-297.

Wolff, A.C., Hammond M.E.H., Hicks D.G., Dowsett M., McShane L.M., Allison K.H., Allred D.C., Bartlett J.M.S., Bilous M., Fitzgibbons P., Hanna W., Jenkins R.B., Mangu P.B., Paik S., Perez E.A., Press M.F., Spears P.A., Vance G.H., Viale G. and Hayes D.F. (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. 31, 3997-4014.

Wu, Y., Chen L., Cao L., Sheng W. and Yang B.B. (2004). Overexpression of the C-terminal PG-M/versican domain impairs growth of tumor cells by intervening in the interaction between epidermal growth factor receptor and β1-integrin. J. Cell Sci., 117, 2227-2237.

Wu Y.J., La Pierre D.P., Wu J., Yee A.J. and Yang B.B. (2005). The interaction of versican with its binding partners. Cell Res. 15, 483-494.

Yang X.Q., Chen C., Peng C.W., Hou J.X., Liu S.P., Qi C.B., Gong Y.P., Zhu X.B., Pang D.W. and Li Y. (2011). Quantum dot-based quantitative immunofluorescence detection and spectrum analysis of epidermal growth factor receptor in breast cancer tissue arrays. Int. J. Nanomedicine 6, 2265-2273.

Yee A.J.M., Akens M., Yang B.L., Finkelstein J., Zheng P.S., Deng Z. and Yang B. (2007). The effect of versican G3 domain on local breast cancer invasiveness and bony metastasis. Breast Cancer Res. 9, 1-11.

Zhang Y., Cao L., Yang B.L. and Yang B.B. (1998). The G3 domain of versican enhances cell Proliferation via epidermial Growth Factor-like Motifs. J. Biol. Chem. 273, 21342-21351.

Table 1. Clinicopathological characteristics of canine mammary benign mixed tumours (BMTs), carcinomas in mixed tumours (CMTs) and carcinosarcomas (CSs).

Parameters	**BMT** n/total (%)	**CMT** n/total (%)	**CS** n/total (%)	***P value***
Mean age	8.72	10.24	11.9	*0.0036**
Size				
≤ 3cm	15/18 (83.3%)	10/39 (25.6%)	0/15 (0%)	*< 0.0001**
3 < x ≤ 5 cm	1/18 (5.6%)	21/39 (53.9%)	5/15 (33.3%)	
> 5 cm	2/18 (11.1%)	8/39 (20.5%)	10/15 (66.7%)	
Lymph node Metastasis				
Negative	-	36/38 (94.7%)	9/14 (69.2%)	*0.0134§*
Positive	-	2/38 (5.3%)	5/14 (30.8%)	
Pulmonary metastases				
Negative	-	38/41 (92.7%)	12/17 (70.6%)	*0.0258§*
Positive	-	3/41 (7.3%)	5/17 (29.4%)	
Clinical staging				
I	-	9/39 (23.1%)	0/0%	*0.7011§*
II	-	18/39 (46.2%)	5/16 (31.2%)	
III	-	7/39 (17.9%)	3/16 (18.8%)	
IV	-	2/39 (5.1%)	3/16 (18.8%)	
V	-	3/39 (7.7%)	5/16 (31.2%)	
Histological grade				
I	-	31/41 (75.6%)	3/18 (16.7%)	*< 0.0001§*
II	-	9/41 (22%)	7/18 (38.9%)	
III	-	1/41 (2.4%)	8/18 (44.4%)	

***** Kruskal Wallis test. § Mann–Whitney U test. The values were considered significant when $P<0.05$.

Table 2. Immunoreactivity for versican, EGFR, HER-2, HER-3 and CD44 in canine mammary benign mixed tumours (BMTs), carcinomas in mixed tumours (CMTs) and carcinosarcomas (CSs).

		BMT		CMT		CS	
		N	B	IS	IN	IS	IN
		n/total (%)	n/total (%)	n/total (%)	n/total (%)	n/total (%)	n/total (%)
Versican (Median)		1	90	140	280	120	180
EGFR							
	0	3/11 (27.3%)	0/16 (0%)	0/38 (0%)	2/38 (5.3%)	1/12 (8.3%)	1/16 (6.3%)
	1	8/11 (72.7%)	9/16 (56.3%)	1/38 (2.6%)	16/38 (42.1%)	2/12 (16.7%)	4/16 (25%)
	2	0/11 (0%)	7/16 (43.7%)	13/38 (34.2%)	16/38 (42.1%)	2/12 (16.7%)	10/16 (62.4%)
	3	0/11 (0%)	0/16 (0%)	24/38 (63.2%)	4/38 (10.5%)	7/12 (58.3%)	1/16 (6.3%)
HER-2							
	0	1/13 (7.7%)	2/16 (12.5%)	0/37 (0%)	5/39 (12.8%)	0/11 (0%)	2/18 (11.1%)
	1	7/13 (53,8%)	3/16 (18.8%)	6/37 (16.2%)	20/39 (51.3%)	2/11 (18.2%)	10/18 (55.6%)
	2	4/13 (30.8%)	10/16 (62.5%)	22/37 (59.5%)	10/39 (25.6%)	9/11 (81.8%)	6/18 (33.3%)
	3	1/13 (7.7%)	1/16 (6.2%)	9/37 (24.3%)	4/39 (10.3%)	0/11 (0%)	0/18 (0%)
HER-3							
Cytoplasmic							
	0	3/16 (18.8%)	1/16 (6.3%)	2/35 (5.7%)	4/34 (11.8%)	2/14 (14.3%)	4/17 (23.5%)
	1	11/16 (68.8%)	9/16 (56.3%	25/35 (71.4%)	22/34 (64.7%)	10/14 (71.4%)	12/17 (70.6%)
	2	2/16 (12.4%)	6/16 (37.4%)	8/35 (22.9%)	8/34 (23.5%)	2/14 (14.3%)	1/17 (5.9%)
Nuclear							
	0	9/16 (56.3%)	7/16 (43.8%)	24/35 (68.6%)	28/34 (82.4%)	11/14 (78.6%)	15/17 (88.2%)
	1	6/16 (37.4%)	7/16 (43.8%)	9/35 (25.7%)	5/34 (14.7%)	3/14 (21.4%)	2/17 (11.8%)
	2	1/16 (6.3%)	2/16 (12.4%)	2/35 (5.7%)	1/34 (2.9%)	0/14 (0%)	0/17 (0%)
Membrane							
	0	15/16 (93.8%)	14/16 (87.5%)	31/35 (88.6%)	31/34 (91.2%)	14/14 (100%)	17/17 (100%)
	1	1/16 (6.2%)	2/16 (12.5%)	4/35 (11.4%)	3/34 (8.8%)	0/14 (0%)	0/17 (0%)
	2	0/16 (0%)	0/16 (0%)	0/35 (0%)	0/34 (0%)	0/14 (0%)	0/17 (0%)
CD44							
	< 3	8/13 (61.6%)	2/17 (11.8%)	3/38 (7.9%)	3/39 (7.4%)	2/10 (20%)	1/14 (7.1%)
	4—6	5/13 (38.4%)	15/17 (88.2%)	14/38 (36.8%)	22/39 (56.5%)	6/10 (60%)	8/14 (57.2%)
	7—9	0/13 (0%)	0/17 (0%)	16/38 (42.1%)	13/39 (33.5%)	2/10 (20%)	5/14 (35.7%)
	≥ 10	0/13 (0%)	0/17 (0%)	5/38 (13.2%)	1/39 (2.6%)	0/10 (0%)	0/14 (0%)

N=normal; B=Benign; IS= in situ; IN = Invasive

Table 3. Clinicopathological characteristics of low (Group 1) and high (Group 2) versican expression of canine mammary carcinomas in mixed tumours (CMTs) and carcinosarcomas (CSs).

	CMT			CS		
	Group 1	**Group 2**	***P value***	**Group 1**	**Group 2**	***P value***
Parameters	n/total (%)	n/total (%)		n/total (%)	n/total (%)	
Lymph node metastasis						
Negative	17/18 (94.4%)	19/20 (95%)	*0.4849*	3/5 (60%)	6/9 (66.7%)	*0.4363*
Positive	1/18 (5.6%)	1/20 (5%)		2/5 (40%)	3/9 (33.3%)	
Pulmonary metastases						
Negative	20/20 (100%)	18/21 (85.7%)	*0.1248*	5/6 (83.3%)	7/11 (63.6%)	*0.2228*
Positive	0/20 (0%)	3/21 (14.3%)		1/6 (16.7%)	4/11 (36.4%)	
Size						
≤ 3cm	3/19 (15.8%)	7/20 (35%)		0/5 (0%)	0/10 (0%)	
3 < x ≤ 5 cm	14/19 (73.7%)	7/20 (35%)	*0.1681*	1/5 (20%)	4/10 (40%)	*0.1855*
> 5 cm	2/19 (10.5%)	6/20 (30%)		4/5 (80%)	6/10 (60%)	
Clinical staging						
I	2/19 (10.5%)	7/20 (35%)				
II	14/19 (73.7%)	4/20 (20%)		1/6 (16.7%)	4/10 (40%)	
III	2/19 (10.5%)	5/20 (25%)	*0.2988*	2/6 (33.3%)	1/10 (10%)	*0.4776*
IV	1/19 (5.3%)	1/20 (5%)		2/6 (33.3%)	1/10 (10%)	
V	0/19 (0%)	3/20 (15%)		1/6 (16.7%)	4/10 (40%)	
Histological grade						
I	16/20 (80%)	15/21 (71.4%)		3/7 (42.9%)	0/11 (0%)	
II	4/20 (20%)	5/21 (23.8%)	*0.2479*	1/7 (14.2%)	6/11 (54.5%)	*0.1756*
III	0/20 (0%)	1/21 (4.8%)		3/7 (42.9%)	5/11 (45.5%)	

Mann Whitney test. The values were considered significant when $P<0.05$.

Table 4. Differences EGFR, HER-2, cytoplasmic HER-3 and CD44 expressions in *in situ* and invasive carcinomatous areas between versican low and high expression groups in CMTs and CSs.

	Carcinomatous areas		**CMT** ***P value***	**CS** ***P value***
EGFR	*In situ*	G1 x G2	*0.1262*	*0.4621*
	Invasive	G1 x G2	*0.1514*	*0.0837*
HER-2	*In situ*	G1 x G2	*0.0136**	*0.3818*
	Invasive	G1 x G2	*0.4273*	*0.0375**
HER-3 Cytoplasmic	*In situ*	G1 x G2	*0.3911*	*0.4646*
	invasive	G1 x G2	*0.0415**	*0.0340**
HER-3 Nuclear	*In situ*	G1 x G2	*>0.9999*	*0.0683*
	Invasive	G1 x G2	*0.4413*	*0.3596*
HER-3 Membrane	*In situ*	G1 x G2	*0.6766*	*>0.9999*
	Invasive	G1 x G2	*0.5455*	*>0.9999*
CD44	*In situ*	G1 x G2	*0.4926*	*0.5000*
	Invasive	G1 x G2	*0.4995*	*0.0474**

Mann Whitney test. *Statistically significant at $P< 0.05$.
(G1= low versican expression; G2= high versican expression).

Fig. 1. Benign mixed tumours in canine mammary gland. (a) Benign epithelial and myoepithelial cells proliferation with mixoid matrix areas. HE, 40x. (b) Low stromal versican expression adjacent to benign epithelial cells proliferation. 60x. (c) Incomplete membrane EGFR staining in benign epithelial cells. 60x. (d) Incomplete membrane HER-2 staining in benign epithelial cells. 60x. (e) Weak cytoplasmic HER-3 expression in benign epithelial cells in benign mixed tumour. 60x. (f) Moderate CD44 expression in benign epithelial cells. 20x. (b-f) Immunohistochemical stain with Mayer's haematoxylin counterstain.

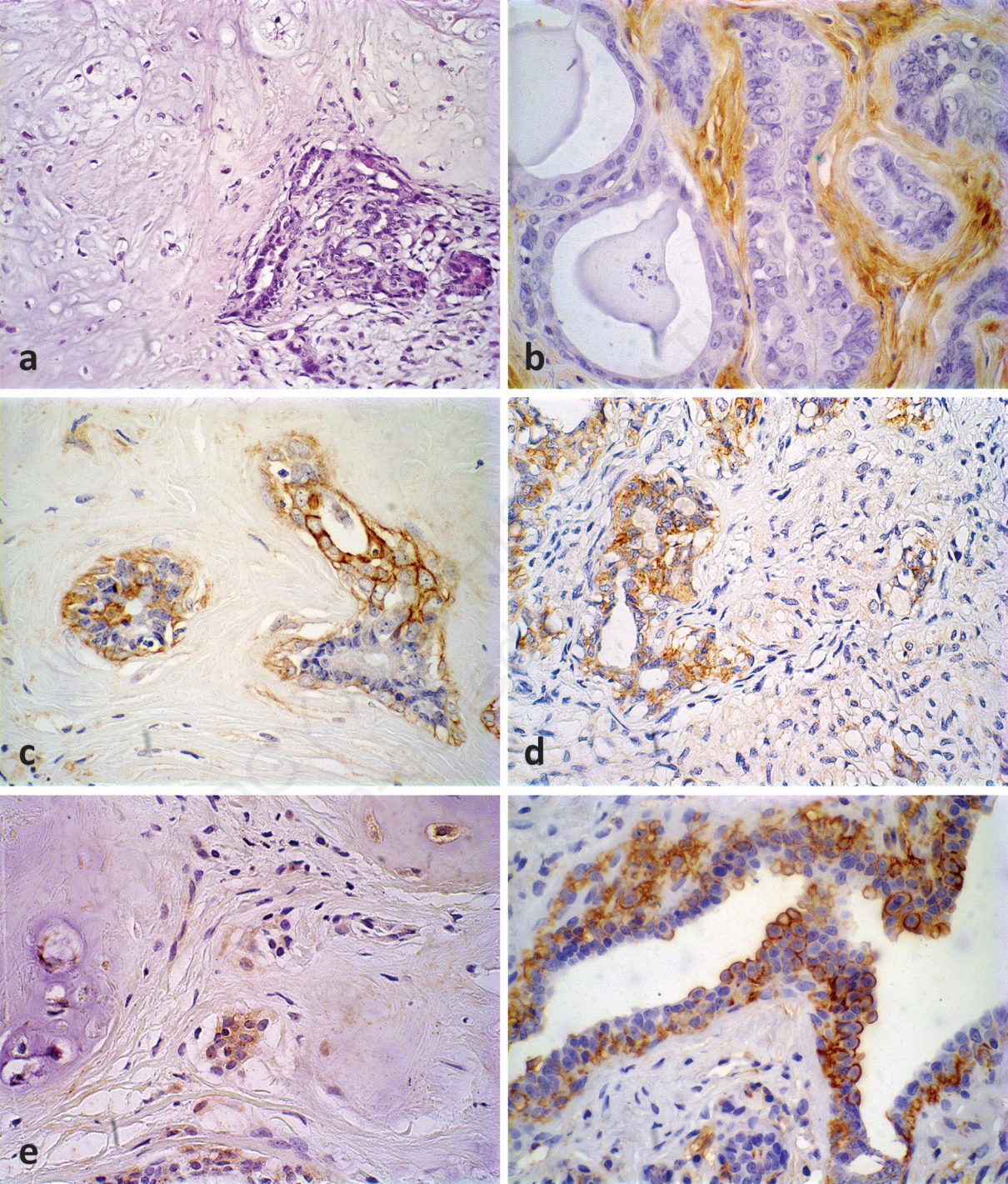
Fig. 2. (a) Versican, (b) EGFR, (c) HER-2 and (d) CD44 expression in benign mixed tumours (BMT), carcinomas in mixed tumours (CMT) and carcinosarcomas (CS). Difference between normal (N) and benign (B) epithelial cells in BMT and between *in situ* (IS) and invasive (IN) areas in CMT and CS.

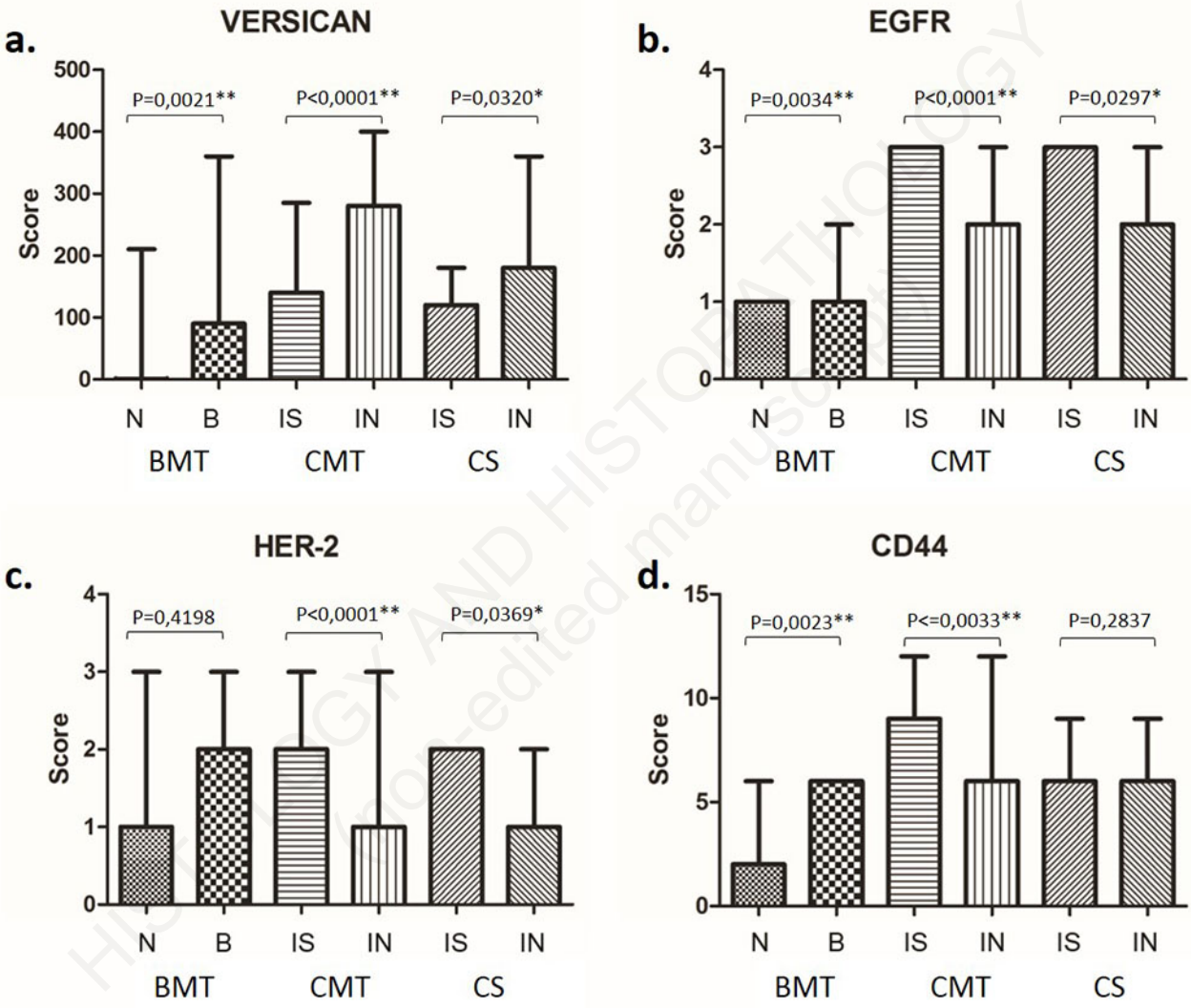
Wilcoxon test. *$p<0.05$ **$p<0.001$

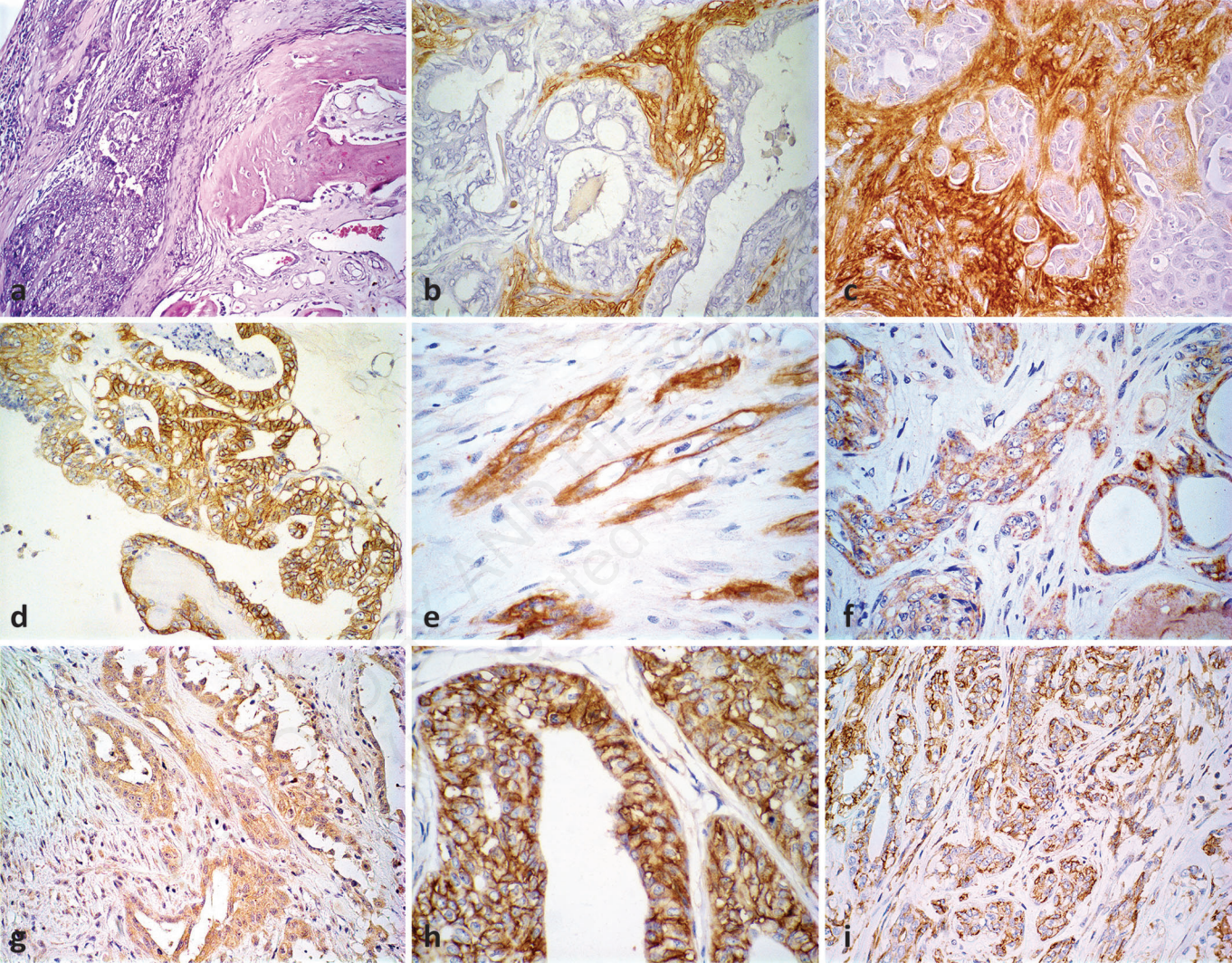
Fig. 3. Carcinoma in mixed tumor in canine mammary gland. (a) Malignant epithelial cells proliferation adjacent to osteoid matrix in carcinoma in mixed tumor of canine mammary gland. HE, 20x. (b) Moderate stromal versican expression adjacent to *in situ* carcinomatous areas. 40x. (c) Strong stromal versican expression adjacent to invasive carcinomatous areas. 40x. (d) Complete membrane EGFR staining in *in situ* carcinomatous areas. 40x. (e) Incomplete membrane EGFR staining in invasive carcinomatous areas. 60x. (f) Incomplete membrane HER-2 staining in invasive carcinomatous areas in carcinoma in mixed tumour. 60x. (g) Weak cytoplasmic HER-3 expression in *in situ* and invasion. 40x. (h) Strong CD44 expression in *in situ* carcinomatous areas. 60x. (i) Moderate to strong CD44 expression in invasive carcinomatous areas. 40x. (b-i) Immunohistochemical stain with Mayer's haematoxylin counterstain.

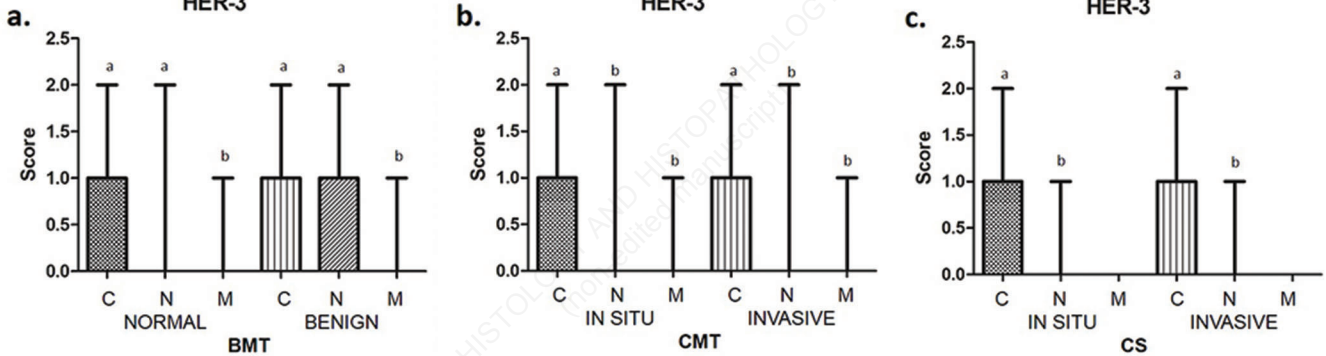
Fig. 4. Cytoplasmic (C), nuclear (N) and membrane (M) HER-3 expression in BMT, CMT and CS. Difference between normal and benign epithelial cells in (a) BMT and between *in situ* and invasive areas in (b) CMT and (c) CS. Different letters demonstrate where statistical differences were present ($P < 0.05$). Wilcoxon test.

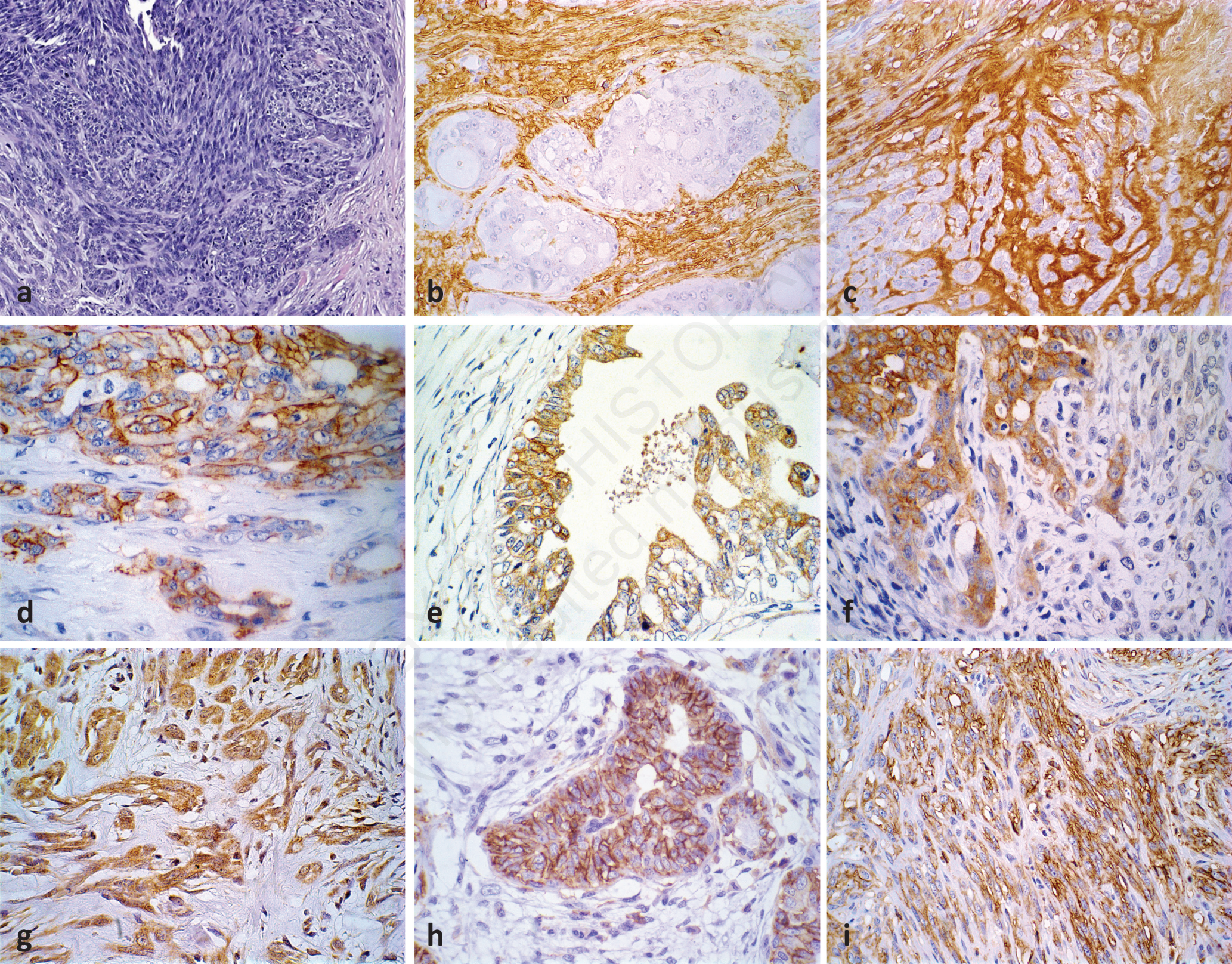
Fig. 5. Carcinosarcoma in canine mammary gland. (a) Malignant spindle epithelial cells proliferation in carcinosarcoma of canine mammary gland. HE, 10x. (b) Moderate stromal versican expression adjacent to *in situ* carcinomatous areas. 40x. (c) Strong stromal versican expression adjacent to invasive areas. 40x. (d) Incomplete membrane EGFR staining in invasive carcinomatous areas. 60x. (e) Incomplete membrane HER-2 staining in *in situ* carcinomatous area. 40x. (f) Increased membrane HER-2 expression in invasive area. 60x. (g) Cytoplasmic HER-3 expression in invasive carcinomatous areas in carcinosarcoma. 60x. (h) Strong CD44 expression in *in situ* carcinomatous areas. 40x. (i) Strong CD44 expression in invasive carcinomatous areas. 40x. (b-i) Immunohistochemical stain with Mayer's haematoxylin counterstain.


a
b
c
d
e


a.
VERSICAN
Score
500
400
300
200
100
0
P=0,0021**
P<0,0001**
P=0,0320*
N
B
IS
IN
IS
IN
BMT
CMT
CS
b.
EGFR
Score
4
3
2
1
0
P=0,0034**
P<0,0001**
P=0,0297*
N
B
IS
IN
IS
IN
BMT
CMT
CS
c.
HER-2
Score
4
3
2
1
0
P=0,4198
P<0,0001**
P=0,0369*
N
B
IS
IN
IS
IN
BMT
CMT
CS
d.
CD44
Score
15
10
5
0
P=0,0023**
P<=0,0033**
P=0,2837
N
B
IS
IN
IS
IN
BMT
CMT
CS


a
b
c
d
e
f
g
h
i


a.
HER-3
Score
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
a
a
b
C
N
M
C
N
M
NORMAL
BENIGN
BMT
b.
HER-3
Score
a
b
b
a
b
b
C
N
M
C
N
M
IN SITU
INVASIVE
CMT
c.
HER-3
Score
a
b
a
b
C
N
M
C
N
M
IN SITU
INVASIVE
CS


a
b
c
d
e
f
g
h
i