ARTICLE IN PRESS

Research in Veterinary Science ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours

B. Restucci *, M. Martano, P. Maiolino

Department of Veterinary Medicine and Animal Production, Naples University "Federico II", Via F. Delpino 1, 80137 Naples, Italy

A R T I C L E   I N F O

Article history:
Received 17 July 2014
Accepted 1 March 2015

Keywords:
Angiogenesis
Mammary tumours
Dog
Endothelin-1
Endothelin-A-receptor

A B S T R A C T

Endothelins and their receptors have been implicated in numerous diseases and have recently emerged as relevant players in a variety of malignancies. Tumours overexpress the Endothelin-1 (ET-1) and the Endothelin-A receptors (ET_AR) and their interaction enhances tumour growth and metastasis by promoting tumour cell survival, proliferation and angiogenesis. In this study we have evaluated the expression of ET-1 and ET_AR in 50 canine mammary tumours, compared to normal controls. Results demonstrated a progressive increase in ET-1 and ET_AR expression from benign tumour to grade 1 and to grade 2 malignant mammary tumours with a decrease of expression in grade 3 carcinomas. Co-localization of ET-1 and ET_AR was observed in benign mammary tumours and in G1 and G2 carcinomas, while absent in G3 carcinomas. Concluding, ET-1/ET_AR can be considered reliable markers for evaluating malignancy of canine mammary tumours and could have importance for the development of specific anticancer therapies.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The Endothelin (ET) family consists of three isopeptides named ET-1, ET-2 and ET-3, which are produced by endothelial and several epithelial cell types, playing a relevant role in cancer biology by intervening and regulating many processes such as aberrant proliferation, escape from apoptosis and angiogenesis (Levin, 1995; Nelson et al., 2003). All these effects are obtained by linking of endothelins with distinct G protein-coupled receptors ET_AR and ET_BR which act in opposite manner and induce divergent intracellular effects (Simonson and Dunn, 1990).

In particular, ET-1 has a high affinity to ET_AR and through this linking can trigger many biological mechanisms such as modulating cell behaviour and local microenvironment homeostasis (Bhalla et al., 2009); yet, the most important role played by the ET axis seems connected to neoplastic transformation and tumour progression (Nakamuta et al., 1993). In fact, ET-1 can stimulate DNA synthesis promoting cell proliferation, it can alter the connexin expression by interfering with cell communication disrupting the intercellular gap junction (Spinella et al., 2003), and it is involved in neoangiogenesis through complex interaction with Vascular Endothelial Growth Factor (VEGF) synthesis (Wulfing et al., 2004).

Elevated expression of ET-1 and ET_AR has been correlated with poor tumour cell differentiation, increase of microvascular density, incidence of metastasis and reduced disease-free survival time in human breast cancer (Wulfing et al., 2003, 2004). Moreover, ET_AR antagonists are successfully used in a number of human anticancer therapy protocols (Bagnato and Natali, 2004; Kandalaft et al., 2009).

For these reasons, molecules of the ET family and in particular ET-1 are considered useful factors in the evaluation of malignancy and in consequence, in the prognosis for several forms of neoplasia, such as ovarian (Bagnato et al., 2005), prostate (Nelson et al., 1995, 1996), colorectal (Asham et al., 2001) and mammary tumours (Alanen et al., 2000; Kojima and Nihei, 1995).

To the best of our knowledge, little is known about the role ETs play in canine neoplasms (Borzacchiello et al., 2010). The aim of the present study was, on one hand, to evaluate the expression of ET-1 and ET_AR in normal and in neoplastic canine mammary tissue by immunohistochemistry, double immunofluorescence staining and western blot analysis, and, on the other, to correlate results with the histological grade of those tumours.

2. Materials and methods

2.1. Samples

Fifty samples of canine mammary tumours and five matched samples of normal mammary gland tissue were collected. Each tissue sample was divided in two parts: one part was fixed in 10% neutral buffered formalin and the other was quick frozen in isopentane/liquid nitrogen. Fixed samples were routinely processed and stained in haematoxylin and eosin. Each sample was classified according to Goldschmidt et al.'s (2011) criteria (Table 1). Malignant tumours

* Corresponding author. Department of Veterinary Medicine and Animal Production, Naples University "Federico II", Via F. Delpino 1, 80137 Naples, Italy. Tel.: +39 081 2536087; fax: +390812536186.

E-mail address: restucci@unina.it (B. Restucci).

http://dx.doi.org/10.1016/j.rvsc.2015.03.008
0034-5288/© 2015 Elsevier Ltd. All rights reserved.

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008

ARTICLE IN PRESS

Table 1
Histological type of canine mammary tumours classified according to Goldschmidt criteria.

Mammary tumours	Number of samples
Benign tumours	
Intraductal papillary adenoma	4
Adenoma-simple	3
Fibroadenoma	3
Total	10
Malignant tumours	
Tubulopapillary carcinoma	10
Tubular carcinoma	18
Solid carcinoma	12
Total	40

were graded independently by two observers, in 15 well (G1), 13 moderately (G2) and 12 poorly differentiated carcinomas, according to the Histological Grading System proposed by Peña et al. (2013).

2.2. Immunoperoxidase staining

Sections were de-paraffinated in xylene, dehydrated in graded alcohols and washed in 0.01 M phosphate buffered saline (PBS). Endogenous peroxidase was blocked with hydrogen peroxide 0.3% in absolute methanol for 30 min. The streptavidin-biotin-peroxidase method (LSAB Kit; Dako, Glostrup, Denmark) was used. Antigen enhancement was performed by pretreating with microwave heating in citrate buffer, pH 6.00, twice for 5 minutes at 750 W. As primary antibodies, monoclonal mouse anti-human Endothelin-1 (clone TR.ET.48.5; *Sigma Aldrich, Milan*) and polyclonal rabbit anti-human Endothelin-A receptor were used *(Sigma Aldrich)*; they were diluted 1:200 in antibody diluent (Dako) and applied overnight at 4 °C. The immunolabelling procedure included negative control sections incubated with normal serum, instead of primary antibody. A mixture of biotinylated anti-mouse, anti-rabbit and anti-goat immunoglobulins (LSAB Kit; Dako), diluted in PBS was used as secondary antibody and was applied for 30 min. After being washed in PBS, the sections were incubated in streptavidin conjugated with horseradish peroxidase in Tris–HCl buffer containing 0.015% sodium azide (LSAB Kit; Dako) for 30 min. In order to visualize immunolabelling, 3′3′-diaminobenzidine tetrahydrochloride was used as a chromogen, with haematoxylin as a counterstain.

2.3. Double colour immunofluorescence staining

The pre-treatment steps were the same as those used for immunoperoxidase labelling and the procedure was the same as that used by the authors in previous studies (Restucci et al., 2007, 2009). The primary mouse monoclonal anti-Endothelin-1 antibody was diluted 1:10 in the same antibody diluent used for immunoperoxidase labelling and applied overnight at 4 °C. Slides were washed three times in PBS and incubated with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse secondary antibody, diluted 1 in 100 in PBS, for two hours at room temperature. After three washes in PBS, anti-ET_AR monoclonal mouse antibody, diluted 1:10, was applied and the sections were again incubated overnight at 4 °C. Slides were washed again three times in PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Chemicon, Germany) diluted 1 in 50 in PBS, for two hours at room temperature. Slides were rinsed with PBS and mounted in Fluorescent Mounting Medium (Dako). For scanning and photography, a laser scanning microscope (LSM 510; Zeiss, Göttingen, Germany) was used. Mouse monoclonal anti-ET-1 antibody bound to TRITC was illuminated at 543 nm and then read with a 560 nm long pass filter. Monoclonal mouse anti-ET_AR bound to FITC was illuminated at 488 nm and read using a 505–560 nm band pass filter. Two-channel frame-by-frame multitracking was used for detection to avoid cross-talk signals. The different frames were scanned separately, with appropriate installation of the optical path for excitation and emission of each scan according to the manufacturer's instructions.

2.4. Western blot analysis

Five frozen samples for each grade of malignancy were chosen. Samples were homogenized in 100 μL of ice-cold hypotonic lysis buffer (10 mm HEPES, 1.5 mm MgCl2, 10 mm KCl, 0.5 mm phenylmethylsulphonyl fluoride, 1.5 μg/mL soybean trypsin inhibitor, pepstatin A 7 μL/mL, leupeptin 5 μ/mL, 0.1 mm benzamidine, 0.5 mm dithiothreitol (DTT)) and incubated at 0 °C for 45 min, as previously described by Izzo et al. (2001). Thereafter, the supernatant was isolated and protein quantification was performed using a BioRad assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Immunoblotting analysis of ET-1 and ET_AR proteins was performed on cytosolic protein fractions isolated from the samples as previously described (Restucci et al., 2007). Equal amounts of protein (100 μg) were mixed with gel loading buffer (50 mm Tris, 10% SDS, 10% glycerol 2-mercaptoethanol, 2 mg bromophenol/mL) at a ratio of 1:1, boiled for 5 min, then separated under reducing conditions in 6% for ET-1 and in 10% for ET_AR SDS-polyacrylamide minigel. The proteins were transferred onto a nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Laboratories). The membranes were blocked by incubation at 4 °C overnight in a high concentration salt buffer (50 mm Trizma base, 500 mm NaCl, 0.05% Tween-20) containing 5% bovine serum albumin. Membranes were then incubated for 2 hours at room temperature with anti-ET-1 (1:200 v:v or anti-ET_AR (1:200 v:v) followed by incubation with specific horseradish peroxidase (HRP)-conjugate secondary antibody (Dako). The immune complexes were developed using enhanced chemiluminescence detection reagents (Amersham, Cologno Monzese, Italy) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The blots were stripped and reprobed against mouse anti-actin antibody (Calbiochem) at 1:5000 to ensure the amounts of protein loading for each sample. The bands of proteins on X-ray film were scanned and densitometric analysis was performed using the public domain NIH Image J program (developed at the U.S. National Institutes of Health; available at http://rsb.info.nih.gov/nih-image/). The protein concentrations were normalized to the actin levels and expressed as the densitometric ratio. Experiments were repeated twice yielding equal results.

2.5. Scoring of immunoreactivity and statistical analysis

The number of positively-labelled cells was established by counting 1.000 cells in at least 20 high-power (×40 objective and ×10 ocular) fields for each sample and results were expressed as percentage and mean and standard deviation. To determine whether ET-1 and ET_AR positive cell numbers (percentage value) differed between normal mammary gland tissue and benign tumours, between benign and malignant tumours and among the differentiation grades of malignant tumours, a Mann–Whitney test was performed, considering statistically significant a p value of less than 0.05. The correlation between ET-1 and ET_AR expression was assessed using s Spearman's rank correlation coefficient test.

3. Results

3.1. Immunohistochemistry

More detailed results are summarized in Table 2.

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008

Table 2

Correlation between ET-1 and $ET_{A}R$ expressed by mean and standard deviation *(X ± SD)* with histological grade.

	Normal mammary tissue (X ± SD)	Benign mammary tumours (X ± SD)	Malignant mammary tumours (X ± SD)		
			Grade 1	Grade 2	Grade 3
	P = 0.01 (Normal vs Benign)	P = 0.001 (Benign vs Malignant)	P = 0.03 (Grade 1 vs Grade 2)	P = 0.01 (Grade 2 vs Grade 3)	
ET-1	119.4 ± 6.8	161.2 ± 14.66	432.2 ± 123.8	591.4 ± 67	312.2 ± 77.6
$ET_{A}R$	76.8 ± 37.2	139.2 ± 26.9	350.2 ± 53.7	554.4 ± 40.9	216.4 ± 60.8
	P = 0.03 (Normal vs Benign)	P = 0.001 (Benign vs Malignant)	P = 0.03 (Grade 1 vs Grade 2)	P = 0.01 (Grade 2 vs Grade 3)	

3.1.1. Normal samples

Strong immunoreactivity for both ET-1 and $ET_{A}R$ antigens was detected in endothelial cells of stromal vessels. Only few epithelial cells of the ducts and lobules showed weak ET-1 cytoplasmic immune labelling, which was confined to the luminal pole. Myoepithelial cells and fibroblasts in the stroma were negative. The receptor ($ET_{A}R$) was detected only in endothelial cells and in a few epithelial cells. The percentage of positive epithelial cells ranged from 11.3 % to 13.7% for ET-1 (cell number median value 119.4 ± 6.8) and from 3.2% to 11.3% for $ET_{A}R$ (cell number median value 76.8 ± 37.2).

3.1.2. Benign tumours

Strong immunoreactivity for both ET-1 and $ET_{A}R$ antigens was detected in endothelial cells of stromal vessels. In all adenomas the percentage of positive epithelial neoplastic cells ranged from 14.4% to 18.6% for ET-1 (cell number median value 161.2 ± 14.6) and from 9.4% to 17.2% for $ET_{A}R$ (cell number median value 139.2 ± 26.9). In general, immunolabelling was weak and restricted to the luminal pole of each epithelial neoplastic cell. Within the stroma any immunolabelling was detected.

3.1.3. Malignant tumours

Strong immunoreactivity for both ET-1 and $ET_{A}R$ antigens was detected in endothelial cells of stromal vessels. In G1 tumours the percentage of epithelial neoplastic positive cells ranged from 28.3% to 57.1% for ET-1 (cell number median value 432.2 ± 123.8) and from 27.3% to 53.1% for $ET_{A}R$ (cell number median value 350.2 ± 53.7). The intensity of immunostaining for both ET-1 and $ET_{A}R$ was strong and polarized to the luminal compartment of the ductal and lobular neoplastic cells (Fig. 1a and b). In some areas, groups of epithelial neoplastic cells with strong immunolabelling, which was uniform and diffuse within the cytoplasm, were identified. Few ET-1 positive fibroblasts and myoepithelial cells were detected. In G2 tumours the percentage of positive epithelial neoplastic cells ranged from 51.7% to 67.9% for ET-1 (cell number median value 591.4 ± 67), and from 48.7 to 64.9% for $ET_{A}R$ (cell number median value 554.4 ± 40.9). Immunolabelling for both ET-1 and $ET_{A}R$ was strong and distributed throughout the cytoplasm (Fig. 1c). Many strongly positive epithelial neoplastic cells were observed in areas of infiltrating cell proliferation (Fig. 1d). Few ET-1 positive fibroblasts and myoepithelial cells were detected.

In G3 tumours the intensity of ET-1 and $ET_{A}R$ immunolabelling was weak and diffused in the cytoplasm of few epithelial neoplastic cells (Fig. 1e). Strongly positive epithelial neoplastic cells were localized only in areas adjacent to foci of necrosis (Fig. 1f). Percentage of positive epithelial neoplastic cells ranged from 22.4% to 41.2% for ET-1 (cell number median value 312.2 ± 77.6) and from 20.4% to 38.2% for $ET_{A}R$ (cell number median value 216.4 ± 60.8). Very few positive fibroblasts and myoepithelial cells were occasionally detected.

3.2. Double colour immunofluorescence staining

3.2.1. Normal samples

TRITC-conjugated ET-1, evident as a green fluorescence, was localized in stromal and endothelial cells and in few epithelial cells. FITC-conjugated $ET_{A}R$, expressed by a red fluorescence, was evident in endothelial cells and only in few epithelial cells. A co-localization of ET-1 and $ET_{A}R$ was observed as yellow fluorescence, only in fibroblasts and endothelial cells.

3.2.2. Benign tumours

Only few epithelial cells showed a co-localization of ET-1 and $ET_{A}R$.

3.2.3. Malignant tumours

Grade 1 carcinomas showed an immunofluorescence staining for $ET_{A}R$ and ET-1 in few epithelial malignant cells, which often was polarized and co-localized (Fig. 2a–c). Grade 2 carcinomas exhibited strong immunofluorescence staining for $ET_{A}R$ and ET-1, often diffuse within the cytoplasm of some epithelial neoplastic cells and colocalized (Fig. 2d–f). In Grade 3 carcinomas weak $ET_{A}R$ immunostaining was seen in very few malignant epithelial neoplastic cells with a cytoplasmic distribution, while ET-1 was expressed by very few malignant epithelial neoplastic cells with a cytoplasmic distribution and also by endothelial and myoepithelial cells. Co-localization was seen only in very few epithelial neoplastic cells (Fig. 2g–i).

3.3. Western blot analysis

Quantitative expression of ET-1 and $ET_{A}R$ was more marked in Grade 2 malignant tumours confirming the immunohistochemical results. Marked expression of ET-1 was evident also in Grade 3 carcinomas (Fig. 3). Densitometric analysis demonstrated a strong expression of ET-1 and $ET_{A}R$ in G2 malignant canine mammary carcinomas and a decreasing expression in G3 carcinomas (Fig. 4).

3.4. Correlation between ET-1 and $ET_{A}R$ expression with histological grade

A progressive increase in ET-1 and $ET_{A}R$ expression from normal mammary gland tissue, over benign tumours and Grade 1 carcinomas was evident up to Grade 2 malignant tumours; on the other hand, in Grade 3 carcinomas, a decrease of both, ET-1 and $ET_{A}R$ expression was found (Graph Line 1). R value was 0.9 in benign tumours, and 1 in the different grades of malignant tumours.

Statistical analysis showed a significant difference in the expression of ET-1 and $ET_{A}R$ between normal mammary gland and benign tumours (P = 0.01 and 0.03 respectively), between benign and malignant tumours (P = 0.001 for both ET-1 and $ET_{A}R$ expression) as well as between the various degrees of malignancies (P = 0.03

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008

ARTICLE IN PRESS

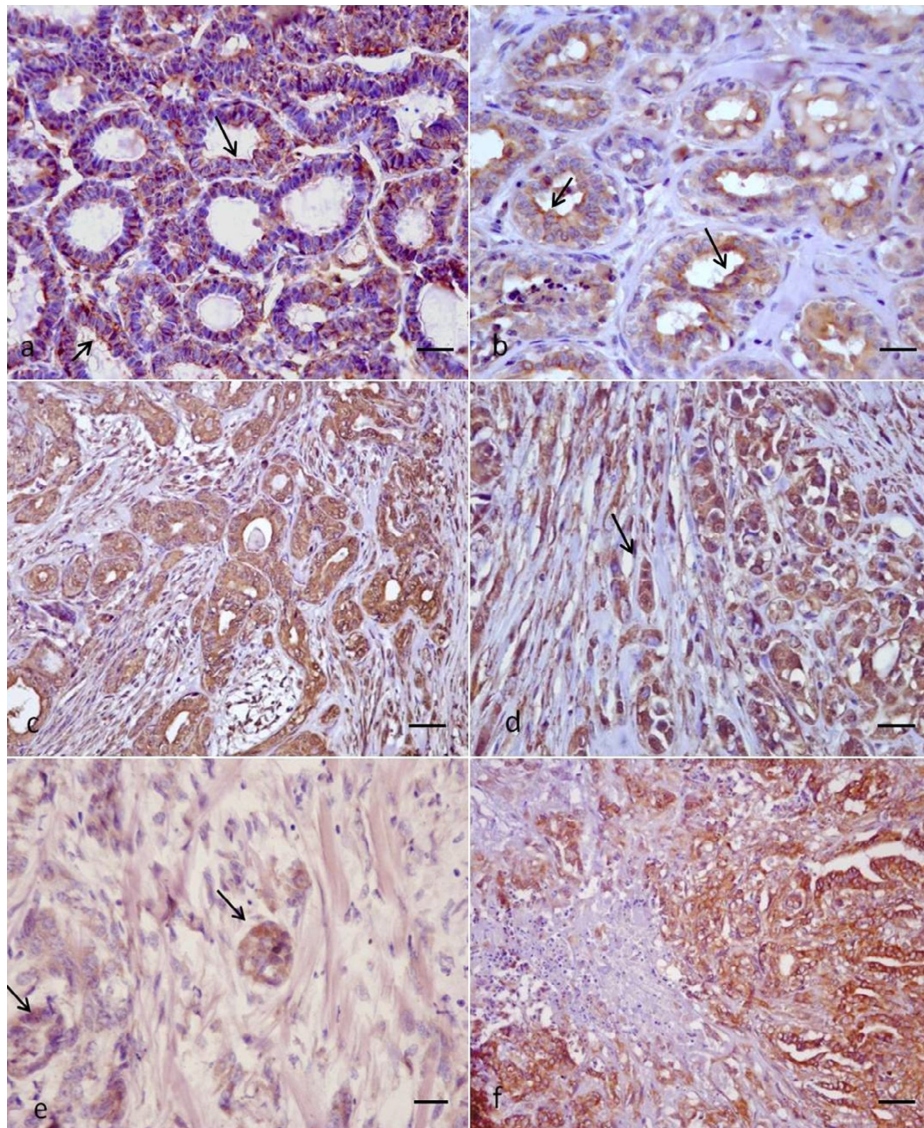


Fig. 1. Streptavidin-biotin-peroxidase stain: (a) Tubular carcinoma Grade 1. Polarized immunostaining for ET-1 is shown by some epithelial cells (33.5%) (arrows). Scale bar = 20 µm. (b) Tubular carcinoma Grade 1. Positivity for ET_AR is evident in some epithelial cells (30.7%) (arrows). Scale bar = 20 µm. (c) Tubular carcinoma Grade 2. Strong immunolabelling for ET-1 diffused in cell cytoplasm is evident in many neoplastic cells (63.8%). Scale bar = 20 µm. (d) Tubular carcinoma Grade 2. Strong immunolabelling for ET_AR is also seen especially in infiltrating cells (62.3%) (arrow). Scale bar = 20 µm. (e) Tubular carcinoma: Grade 3 malignant tumour. Immunolabelling for ET-1 expressed by few neoplastic cells is evident (35%.7) (arrows). Scale bar = 20 µm. (f) Solid carcinoma: Grade 3 malignant tumour. Strong expression of ET-1 is evident near a necrotic area. Scale bar = 20 µm.

between G1 and G2 carcinomas and P = 0.01 between G2 and G3 carcinomas for both ET-1 and ET_AR expression) (Table 2).

4. Discussion

The "angiogenic switch", by which neoplastic cells acquire the ability to provide for their own oxygen and their nutrient requirements, is a key event in malignant transformation and in tumour growth (Hanahan and Folkman, 1996). This vasculogenic potential has already been studied in several spontaneous tumours in animals (Maiolino et al., 2001; Queiroga et al., 2011; Restucci et al., 2000, 2002; Sleeckx et al., 2014; Wolfesberger et al., 2008).

In this study we found a significant increase of ET-1 and its receptor (ET_AR) expression in G1 and G2 mammary carcinomas both by immunohistochemical staining and western blot analysis; this demonstrates an involvement of this protein and its receptor in the early stages of malignant cell transformation and tumour progression when the intensity of cell proliferation and metabolic demands strongly increase.

It has been shown previously that ET-1 is involved in neoplastic proliferation, activating a network of signals that results in the transfer of a mitogen signal to the nucleus, promoting endothelial cell growth (Nelson et al., 2003) which is the first step of the angiogenetic process.

It has also been demonstrated in some tumours, such as in prostate, ovarian and cervical cancer (Bagnato and Rosanò, 2008; Bagnato et al., 1997, 1999; Nelson et al., 1995, 1996; Venuti et al., 2000), that the spontaneous growth of malignant cells was inhibited in the presence of an ET_AR receptor antagonists, which is in further support of ET-1 stimulating cell proliferation, by acting through an autocrine mechanism (Bhalla et al., 2009). Therefore, the ET_AR receptor expression in mammary gland tumours and its progressive intensification in respect to malignant progression, as observed in our set of canine mammary tumours, further confirm this mechanism.

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008

ARTICLE IN PRESS

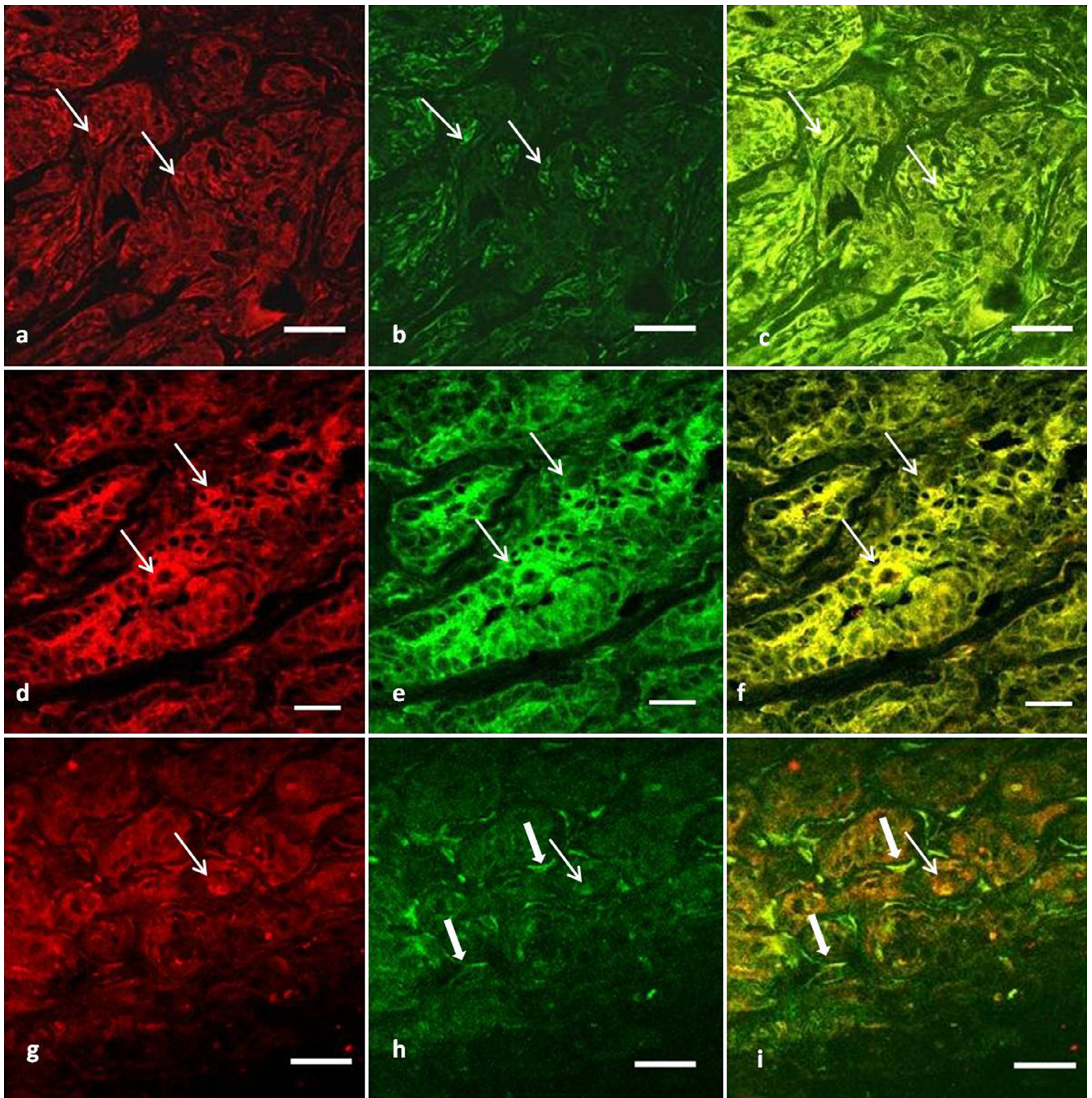


Fig. 2. Two-colour immunofluorescence stain: (a) Tubular carcinoma Grade 1. ET_AR immunostaining corresponding to red TRITC immunofluorescence is evident in few epithelial cells (arrows). Scale bar = 20 µm. (b) Tubular carcinoma Grade 1. ET-1 immunostaining corresponding to green FITC immunofluorescence, with a polarized expression, is evident in few epithelial cells (arrows). Scale bar = 20 µm. (c) Tubular carcinoma Grade 1. Co-expression of ET-1 and ET_AR evident in yellow shows the co-localization of both proteins in few epithelial cells (arrows). Scale bar = 20 µm. (d) Tubular carcinoma Grade 2. Strong ET_AR immunostaining corresponding to red TRITC immunofluorescence is evident in many epithelial cells. Cytoplasmic distribution is evident in some cells (arrows). Scale bar = 20 µm. (e) Tubular carcinoma Grade 2. ET-1 immunostaining corresponding to green FITC immunofluorescence, with strong expression in many cells (arrows). Scale bar = 20 µm. (f) Tubular carcinoma Grade 2. Co-expression of ET-1 and ET_AR evident in yellow shows the co-localization of both proteins in many epithelial cells (arrows). Scale bar = 20 µm. (g) Tubular carcinoma Grade 3. Weak ET_AR immunostaining corresponding to red TRITC immunofluorescence is seen in very few epithelial cells with a cytoplasmic distribution (arrow). Scale bar = 20 µm. (h) Tubular carcinoma Grade 3 ET1 immunostaining evident corresponding to green FITC immunofluorescence is evident in very few neoplastic epithelial cells (arrow) and in endothelial and myoepithelial cells (big arrows). Scale bar = 20 µm. (i) Tubular carcinoma Grade 3. Co-expression of ET-1 and ET_AR evident in yellow, shows the co-localization of both proteins in very few neoplastic epithelial cells (arrow) while in endothelial and myoepithelial cells only ET-1 was evident (big arrows). Scale bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008

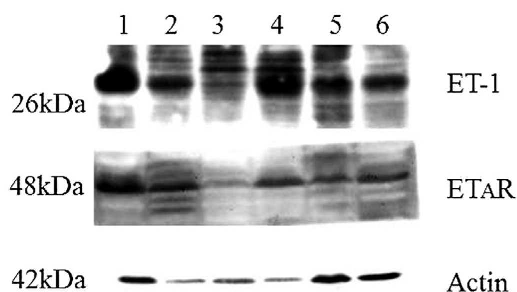


Fig. 3. Western blot for ET-1, ET_AR. Actin protein levels were detected to ensure protein loading and allow normalization. Line 1: Benign tumour; Line 2 and Line 3: Grade 1 malignant tumours; Line 4: Grade 2 malignant tumour; Lines 5 and 6: Grade 3 malignant tumours. A marked expression of ET-1 and ET_AR is evident in line 4.

In addition, the interaction ET-1/ET_AR increases the expression of matrix metalloproteinases (MMPs), modulating the invasive capacity of some tumour cells (Rosanò et al., 2001) and can phosphorylate connexin 43, thus altering the gap junction intercellular connections (GJIC) (Spinella et al., 2003). Destabilization of GJIC has been associated with decrease in intercellular communication and the acquisition of a local invasive and metastatic phenotype in human (Carystinos et al., 2002) and animal tumours (Torres et al., 2005).

An increased ET-1/ET_AR expression is positively correlated with vascular endothelial growth factor expression (VEGF) and with angiogenesis in different malignancies including those of human (Wulfing et al., 2003) and canine mammary gland tumours (Martano et al., 2008). In particular ET-1 can stimulate endothelial cell proliferation and can induce the production of VEGF (Salani et al., 2000), which in turn stimulates angiogenesis and tumour growth, respectively, through paracrine and autocrine mechanisms (Restucci et al., 2004).

In human ovarian neoplasia a correlation between the increase of ET-1 and cyclooxygenase-2 (COX-2) was also shown (Spinella et al., 2004). COX-2 induces the synthesis of PGE2 and VEGF, confirming both, the link between ET-1 and VEGF, and the involvement of ET-1 in neoplastic angiogenesis.

Our results clearly show an increase in ET-1 and its receptor expression, hand in hand with an increase in malignancy up to the Grade 2 tumours; it was therefore surprising to observe a decrease in these expressions in G3 neoplasms, in which strong

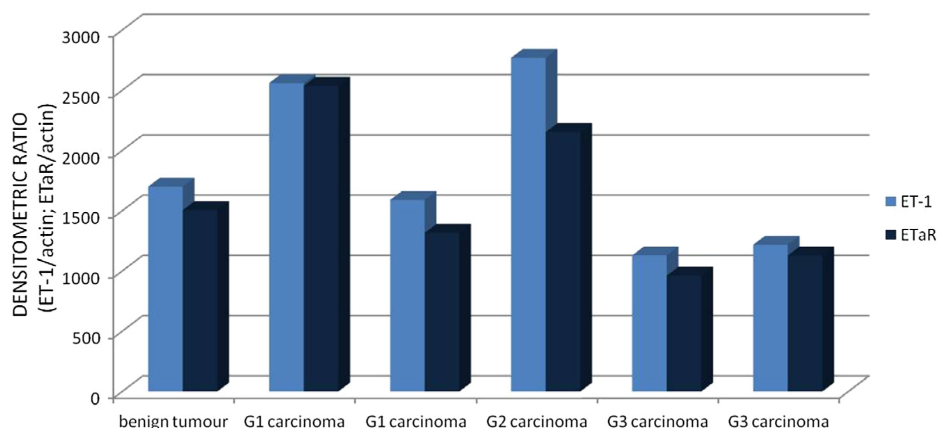
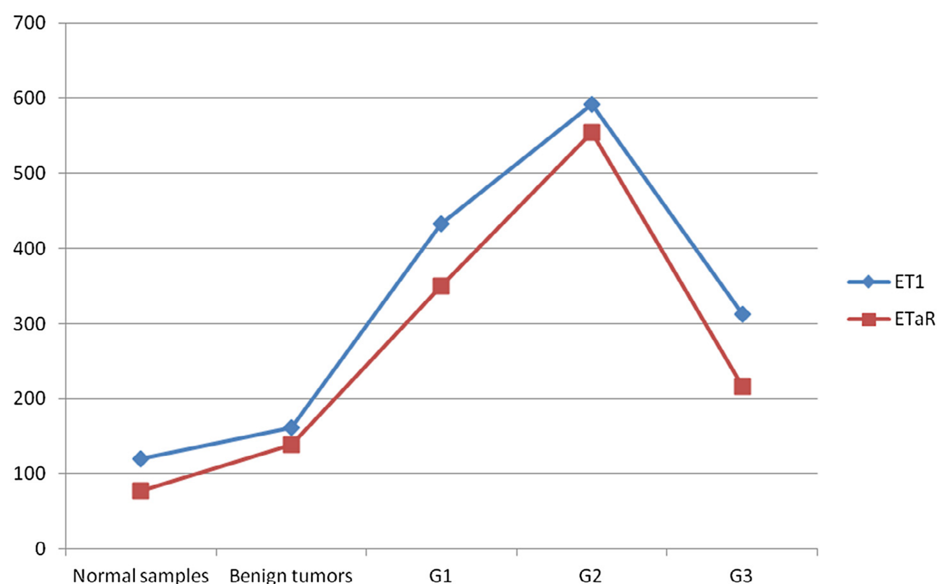


Fig. 4. Quantification of ET-1 and ET_AR protein expression by the ET-1/Actin ratio or ET_AR/Actin ratio. Results of densitometry demonstrate a strong expression in G2 carcinomas and a decreasing expression in G3 carcinomas.



Graph Line 1. Correlation between ET-1 and ET_AR immunolabelled cells (expressed by median value) in normal and neoplastic canine mammary samples.

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008

ARTICLE IN PRESS

immunohistochemical positivity was only found in epithelial neoplastic cells adjacent to necrotic areas.

This feature was apparently in contrast to western blot results, in which an increase of ET1 was evidenced in some G3 carcinomas, and could be explained by the strong ET1 expression of the epithelial neoplastic cells located near to necrotic areas but also by the same expression of endothelial cells and fibroblasts.

This relative lack of ET-1 expression in highly malignant epithelial cells could be explained by marked cellular anaplasia and cellular dysfunction; we also suggest that the high levels of VEGF, which have been evidenced in highly malignant mammary tumours (Restucci et al., 2002) may suppress ET-1 synthesis via a negative feed-back mechanism.

In addition, enhanced ET-1 expressions by those neoplastic cells which are located near to necrotic areas, in G2 and mostly in G3 carcinomas, strongly suggest a positive feedback between tissue hypoxia and ET-1. Thus, this confirms that hypoxia is a potent angiogenetic stimulator and that it can indirectly enhance neoplastic growth and invasiveness through ET-1 release. This highlights the link between hypoxia and the increased invasiveness in malignant tumours through the synthesis of angiogenic cytokines (Shweiki et al., 1992). It has been demonstrated in human breast cancer that invasiveness may be reduced by selective ET_AR antagonism, thereby confirming the ET-1 involvement; this emphasizes the potential therapeutic usefulness of ET_AR antagonists (Smollich et al., 2008).

Therefore, elucidating further the cellular mechanisms triggered by ET-1/ET_AR interaction may open an new avenue to develop chemotherapy protocols for canine mammary tumours; yet, not only, we also believe that ET-1/ET_AR expression in spontaneous canine mammary tumour may add additional interest in this animal model for improving human breast cancer therapy.

References

Alanen, K., Deng, D.X., Chakrabarti, S., 2000. Augmented expression of endothelin-1, endothelin-3 and endothelin-B receptor in breast carcinoma. Histopathology 36, 161–167.

Asham, E., Shankar, A., Loizidou, M., Fredericks, S., Miller, K., Boulos, P.B., et al., 2001. Increased endothelin-1 in colorectal cancer and reduction of tumor growth by ET (A) receptor antagonism. British Journal of Cancer 85, 1759–1763.

Bagnato, A., Natali, P.G., 2004. Endothelin receptors as novel targets in tumor therapy. Journal of Translational Medicine 2, 16–24.

Bagnato, A., Rosanò, L., 2008. The endothelin axis in cancer. International Journal of Biochemistry and Cell Biology 40, 1443–1451.

Bagnato, A., Tecce, R., Di Castro, V., Catt, K.J., 1997. Activation of mitogenic signaling by endothelin-1 in ovarian carcinoma cells. Cancer Research 57, 1306–1311.

Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J.R., Tecce, R., Nicotra, M.R., et al., 1999. Expression of endothelin 1 and Endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. Cancer Research 59, 720–727.

Bagnato, A., Sinella, F., Rosano, L., 2005. Emerging role of the endothelin axis in ovarian tumor progression. Endocrine-Related Cancer 12, 761–772.

Bhalla, A., Haque, S., Taylor, I., Winslet, M., Loizidou, M., 2009. Endothelin receptor antagonism and cancer. European Journal of Clinical Investigation 10, 1365–2362.

Borzacchiello, G., Mogavero, A., Tortorella, G., Catone, G., Russo, M., 2010. Expression of endothelin-1 and endothelin receptor A in canine ovarian tumours. Reproduction in Domestic Animals 45, 465–468.

Carystinos, G.D., Bier, A., Batist, G., 2002. The Role of connexin-mediated cell-cell communication in breast cancer metastasis. Journal of Mammary Gland Biology and Neoplasia 6, 431–440.

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

Hanahan, D., Folkman, J., 1996. Patterns and emerging mechanism of the angiogenesis switch during tumorigenesis. Cell 86, 353–364.

Izzo, A.A., Fezza, F., Capasso, R., Bisogno, T., Pinto, L., Iuvone, T., et al., 2001. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. British Journal of Pharmacology 134, 563–570.

Kandalaft, L.E., Facciabene, A., Buckanovich, R.J., Coukos, G., 2009. Endothelin B receptor, a new target in cancer immune therapy. Clinical Cancer Research 15, 4521–4528.

Kojima, K., Nihei, Z., 1995. Expression of endothelin 1 immunoreactivity in breast cancer. Surgical Oncology 4, 309–315.

Levin, E.R., 1995. Endothelins. The New England Journal of Medicine 333, 356–363.

Maiolino, P., Papparella, S., Restucci, B., De Vico, G., 2001. Angiogenesis in squamous cell carcinomas of canine skin: an immunohistochemical and quantitative analysis. Journal of Comparative Pathology 125, 117–121.

Martano, M., Restucci, B., Mogavero, S., Maiolino, P., 2008. Espressione della endotelina 1 e del recettore A in tumori mammari di cagna. Indagini preliminari. Associazione Italiana di Patologia Veterinaria 9–14.

Nakamuta, M., Ohashi, M., Tabata, S., Tanabe, Y., Goto, K., Naruse, M., 1993. High plasma concentration of Endothelin-like immunoreactivities in patients with hepatocellular carcinoma. The American Journal of Gastroenterology 88, 248–252.

Nelson, J.B., Hedican, S.P., George, D.J., Reddi, A.H., Paiantadosi, S., Eisenberger, M.A., et al., 1995. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nature Medicine 1, 944–949.

Nelson, J.B., Chan-Tack, K., Hedican, S.P., Magnuson, S.R., Opgenorth, T.J., Bova, G.S., et al., 1996. Endothelin-1 production and decreased Endothelin B receptor expression in advanced prostate cancer. Cancer Research 57, 35–37.

Nelson, J.C., Bagnato, A., Battistini, B., Nisen, P., 2003. The endothelin axis: emerging role in cancer. Nature Reviews Cancer 3, 110–116.

Peña, L., De Andrés, P.J., Clemente, M., Cuesta, P., Pérezz-AlenzaM, D., 2013. Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. Veterinary Pathology 50, 94–105.

Queiroga, F.L., Pires, I., Parente, M., Gregório, H., Lopes, C.S., 2011. COX-2 over-expression correlates with VEGF and tumour angiogenesis in canine mammary cancer. The Veterinary Journal 189, 77–82.

Restucci, B., De Vico, G., Maiolino, P., 2000. Evaluation of angiogenesis in canine mammary tumors by quantitative platelet endothelial cell adhesion molecule immunohistochemistry. Veterinary Pathology 37, 297–301.

Restucci, B., Papparella, S., Maiolino, P., De Vico, G., 2002. Expression of vascular endothelial growth factor (VEGF) in canine mammary tumors. Veterinary Pathology 39, 488–493.

Restucci, B., Borzacchiello, G., Maiolino, P., Martano, M., Paciello, O., Papparella, S., 2004. Expression of vascular endothelial growth factor receptor flk-1 in canine mammary tumours. Journal of Comparative Pathology 130, 99–104.

Restucci, B., Maiolino, P., Martano, M., Esposito, G., De Filippis, D., Borzacchiello, G., et al., 2007. Expression of β-catenin, E-cadherin and APC in canine mammary tumors. Anticancer Research 27, 3083–3090.

Restucci, B., Martano, M., De Vico, G., Lo Muzio, L., Maiolino, P., 2009. Expression of E cadherin, β-catenin and APC protein in canine colorectal tumours. Anticancer Research 29, 2919–2926.

Rosanò, L., Varmi, M., Salani, D., Di Castro, V., Spinella, F., Natali, P.G., et al., 2001. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. Cancer Research 61, 8340–8346.

Salani, D., Taraboletti, G., Rosano, L., Di Castro, V., Borsotti, P., Giavazzi, R., et al., 2000. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. American Journal of Pathology 157, 1703–1711.

Shweiki, D., Itin, A., Soffer, D., Keshet, E., 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843–845.

Simonson, M., Dunn, M., 1990. Cellular signaling by peptides of the endothelin gene family. The FASEB Journal 4, 2989–3000.

Sleeckx, N., Van Brantegem, L., Van den Eynden, G., Fransen, E., Casteleyn, C., Van Cruchten, S., et al., 2014. Angiogenesis in canine mammary tumours: a morphometric and prognostic study. Journal of Comparative Pathology 150, 175–183.

Smollich, M., Gotte, M., Kersting, C., Fischgrabe, J., Kiesel, L., Wulfing, P., 2008. Selective ETAR antagonist inhibits hypoxia-induced breast cancer cell invasion. Breast Cancer Research Treatment 1089, 175–182.

Spinella, F., Rosanò, L., Di Castro, V., Nicotras, M.R., Natali, P.G., Bagnato, A., 2003. Endothelin-1 decreases gap junction intercellular communication by inducing phosphorylation of Connexin 43 in human ovarian carcinoma cells. The Journal of Biological Chemistry 17, 41294–41301.

Spinella, F., Rosanò, L., Di Castro, V., Nicotras, M.R., Natali, P.G., Bagnato, A., 2004. Endothelin-1-induced prostaglandin E2-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. Journal of Biological Chemistry 279, 46700–46705.

Torres, L.N., Matera, J.M., Vasconcellos, C.H., Avanzo, J.L., Hernandez-Blazquez, F.J., Dagli, M.L., 2005. Expression of connexin 26 and 43 in canine hyperplastic and neoplastic mammary glands. Veterinary Pathology 42, 633–641.

Venuti, A., Salani, D., Manni, V., Poggiali, F., Bagnato, A., 2000. Expression of endothelin 1 and endothelin A receptor in HPV-associated cervical carcinoma: new potential targets for anticancer therapy. The FASEB Journal 14 (14), 2277–2283.

Wolfesberger, B., Tonar, Z., Witter, K., Guija de Arespacohaga, A., Skalicky, M., Walter, I., et al., 2008. Microvessel density in normal lymph nodes and lymphomas of dogs and their correlation with vascular endothelial growth factor expression. Research in Veterinary Science 85, 56–61.

Wulfing, P., Raihanatou, D., Kersting, C., Wulfing, C., Poremba, C., Kiesel, L., 2003. Expression of endothelin-1, endothelin-A and endothelin-B receptor in human breast cancer and correlation with long-term follow-up. Clinical Cancer Research 9, 4125–4231.

Wulfing, P., Wersting, C., Tio, J., Fischer, R.J., Wulfing, C., Poremba, C., 2004. Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. Clinical Cancer Research 10, 2393–2400.

Please cite this article in press as: B. Restucci, M. Martano, P. Maiolino, Expression of endothelin-1 and endothelin-1 receptor A in canine mammary tumours, Research in Veterinary Science (2015), doi: 10.1016/j.rvsc.2015.03.008