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HER2/p53 ratios among canine mammary tumours and respective derived cell culture

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Abstract In this study, the expression of p53 and human epidermal growth factor receptor 2 (HER2) proteins were investigated in different canine mammary tumors (CMT) and cell cultures derived from them, for the purpose of determining neoplastic behavior *in vitro*. For this purpose, immunohistochemistry and immunocytochemistry were performed and results were compared according to histological diagnose. CMT were collected and divided into two halves, one for histological and immunohistochemistry analyses and another for cell cultures. Cell markers p53 and HER2 proteins were detected by immunohistochemistry in the original tumors and immunocytochemistry in CMT-derived cell lines. A total of CMT (n = 17) were classified and divided into benign mixed tumor (BMT), complex carcinoma (CC), simple carcinoma (SC), and squamous cell carcinoma (SCC). Low expression of p53 was verified in BMT and CC, averagely in SC and undetectable in SCC. *In vitro*, there was an increase in p53 expression by cultured cells. However, HER2 protein expression was not significant in BMT, CC and SCC. In contrast, MCT-derived cells were considered HER2-positive in all MCT types. In conclusion, the *in vitro* model is essential for the study of neoplastic behavior and understanding of the carcinogenic mechanisms that occur *in vivo*.

Keywords: *In vitro* culture, cancer markers, cell phenotype

1. Introduction

Canine mammary tumours (CMT) are the most common neoplasms in intact female dogs (Dagli 2008; Shafiee et al 2008; Sleeckx et al 2011). Most CMT are of epithelial origin (simple adenoma/simple carcinoma [SC]), some consisting of both epithelial and myoepithelial tissues (complex carcinoma [CC]), a few tumors are of mesenchymal origin (fibroadenoma, fibrosarcoma, osteosarcoma, or others sarcomas), and they are frequently a combination of epithelial and mesenchymal tissues (mixed benign tumours/carcinosarcomas), according to World Health Organization (WHO) classification (Goldschmidt et al 2011). The carcinomas constitute 40–45% of the malignant tumors in dogs (Sleeckx et al 2011). Moreover, mammary tumors with the described complex morphology only seem to exist in dogs and humans, a fact that establishes spontaneous mammary cancers in dogs an excellent model for human breast cancer study (Shafiee et al 2008).

Cell lines obtained from tumors and tissues represent an important means of studying the cancer cells' behavior (Lacroix and Leclerq 2004). Several methods have been described to grow neoplastic breast tissue for both dog and human mammary cancer cells (Hellmén et al 2000; Lacroix and Leclerq 2004). However, success in propagating CMT-derived cell culture demands special care and has been limited in dog species (Król et al 2009; Pawlowski et al 2009; Król et al 2012). The development of cancer *in vivo* involves the aberrant accumulation of cells caused by excessive proliferation, insufficient apoptosis of cells, or dysfunction of cellular differentiation. The protein called p53, encoded by the tumor suppression gene *p53*, functions as a master regulator of both cell division and apoptosis (Lee et al 2004). The proto-oncogene *ERBB2* is located on human chromosome 17 and encodes the 185-kDa transmembrane glycoprotein human epidermal growth factor receptor 2 (HER2), also known as neu or c-erbB-2, which belongs to the HER-2 tyrosine kinase receptor family (Ackland et al 2003; Hsu et al 2009).

In CMT, it has been verified that HER2 amplification and overexpression can be found in several canine and human tissues (Hayden et al 2011; Kim et al 2011). Human breast cancer has been described to express HER-2 positively; it is used as a prognostic marker on its own or in association with other proteins, such p53 (Linjawi et al 2004). The relevance of HER2/p53 overexpression in CMT and derived cultured cell lines as a prognostic factor has not yet been clearly determined in veterinary oncology until now. In order to bring new insights on cancer cells' behavior *in vitro*, the present study aims to determine the

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HER2/p53 expression in different types of CMT and derived cell cultures by immunohistochemistry (IHC) and immunocytochemistry assays.

2. Materials and Methods

A total of 17 tumor samples from 17 female dogs with spontaneous breast cancers were provided during the elective mastectomy procedures by the Faculty of Veterinary Medicine at the University of São Paulo State, Araçatuba, Brazil. A representative fragment of the tumor, which was more than 2.5 cm in diameter, was collected and divided into two halves. One half was minced into small pieces and enzymatically dissociated in RPMI 1640 (1:1) media (Invitrogen®, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen®), 300 UI/ml collagenase (Sigma-Aldrich®, St. Louis, MO, USA), antibiotic-antimycotic solution 100x (Invitrogen®), and 5% of 200 nM glutamine (Invitrogen®). The suspensions with incompletely dissociated tissue were centrifuged at 2.4 *x g* for 5 min and washed twice in fresh RPMI 1640 media plus antibiotic-antimycotic solution 100 x (Invitrogen®) to proceed cell culture. The other half was subjected to histological analysis. Tissue samples were fixed in 10% formalin for 12–14 h. The fixed tissues were embedded in paraffin, then sections of 4 µm thicknesses were cut and stained with hematoxylin-eosin (HE) for histological classification of each tumor used in this study. Unstained sections were kept for immunohistochemistry analysis of cellular markers.

Representative portions of each CMT were fixed in 10% buffered formalin, embedded in paraffin wax, and routinely processed (Andrade et al 2010). The 4-µm tissue sections prepared from each tumor paraffin block were stained with HE. Lesions were classified according to WHO classification, and tumors displaying multiple features were classified according to the most pronounced histological differentiation as: BMT-benign mixed tumor, CC-complex Carcinoma, SC-simple carcinoma, SCC- squamous cell carcinoma (Midsorp et al 1999).

The paraffin-embedded tumor samples were cut to 4 µm thickness and subjected to IHC assay. The antibodies used were p53 mutant (Sigma-Aldrich®) and HER2 (A0485) diluted at 1:350 (DakoCytomation® CA, USA). The standard avidin-biotin-peroxidase complex (ABC, DakoCytomation®) method was used. Unstained sections were deparaffinized, rehydrated, and washed in buffered saline with 0.1% Tween 80. Antigen retrieval was carried out in the microwave with citrate buffer (pH 6.1) for 15 min at 700W, normally damaged by formaldehyde fixation. Before staining, the slides were treated three times with 50% hydrogen peroxide for 30 min to inactivate the endogenous peroxidase and were then rinsed five times in buffered saline for 10 min each to remove remainders. The subsequent step consisted of blocking nonspecific binding by incubating in 15% reconstituted dry skim milk for 90 min. The slides were overlaid by 200 µl of diluted antibody overnight at 4 °C in a humidified atmosphere. After washes with buffered saline, 200 µl of the secondary antibody was incubated was added to each slide and incubated for 1 h at 37 ºC, and then, 100 µl streptavidin-peroxidase complex (DakoCytomation®) was added to each slide and incubated for 1 h at 37 ºC. In addition, a substrate made fresh in the dark, by mixing equal volumes of 0.02% hydrogen peroxide and 0.6 mg 3,3- diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich®), was added to the slides at room temperature. The reaction was stopped by washing with tap water, and the specific brown color was revealed after counterstaining with Mayer's Haematoxylin. A highly brownish accumulation was taken as control positive, while the absence of the primary antibody was considered as a negative control for the several antibodies. The images were obtained under an AxioImager® A.1 light and ultraviolet microscope connected to AxioCam®MRc (Carl Zeiss, Oberkochen, Germany) and were processed using AxioVision® 4.8 software (Carl Zeiss).

Canine tumor cells and respective tissue pieces were re-suspended in primary culture medium consisting of RPMI 1640 with antibiotic-antimycotic solution 100 x, 10 % FBS, 5% 200 mM glutamine (Invitrogen®), and plated in 25 cm^2 culture flasks (BD Falcon™, Franklin Lakes, NJ, USA) at an initial concentration of 1.5 x 10^4 cells/cm^2 (Figure 1A and 1B). These cells were grown for 3 h at 38.5 °C and 5% of CO_2 in a humidified incubator. After 3 h, the medium was replaced, and the culture conditions were maintained for 24 h. The medium and the tissue fragments were removed and filtered in a cell strainer 70-µm filter (BD Falcon™). At this point, the new medium consisted of F12/Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen®), antibiotic-antimycotic 100 x (Invitrogen®), 10% FBS, and 5% 200 mM glutamine (Invitrogen®). Under this condition, culture flasks were kept in a humidified incubator at 38.5 °C and 5 % CO_2 until complete monolayer confluence. The medium was refreshed every 24 h, and pictures were taken to observe the morphology at 5-day intervals under an inverted microscope (Olympus IX 70, Tokyo, Japan).

Cell staining was performed using the same monoclonal antibodies described for original tumors. Cells were fixed with 4% of paraformaldehyde for 15 min after 48 h of culturing in Lab-Tek® chamber slides (BD Falcon™). The cells were permeabilized for 10 min at room temperature in 0.4% Triton X-100 diluted in phosphate-buffered solution (PBS). The cells were incubated overnight with primary antibodies at 4 °C. After three washes, the cells were incubated with secondary antibody, rabbit anti-mouse, or anti-goat FITC (Sigma-Aldrich®) at a 1:100 dilution. For nuclear staining, DAPI (1 mg/ml) diluted in PBS was added to samples for 15 min. Images were collected under an ultraviolet microscope AxioImager® A.1 light connected to AxioCam®MRc (Carl Zeiss). Counting was processed using AxioVision® 4.8 software (Carl Zeiss).

Data are presented as mean ± standard deviation (SD). Three replicates were performed for each experiment and the results represent these replicates. One-way analysis of variance (ANOVA) for multiple comparisons or two-tailed student *t*-test, whenever applicable, was used. A level of $p < 0.05$ was accepted as significant. The number of p53- and HER2-reactive cells was

quantified by counting cells/mm^2 in a histological and cytological field according to tumor classification using AxioVision® 4.8 software (Carl Zeiss). All analyses were performed 'blind', i.e., the researchers did not know which samples belonged to which experimental group. All statistical analyses were performed using Prism software (GraphPad®, CA, USA).

3. Results

Of the 17 canine mammary tumors collected, five were macroscopically characterized as small nodules and histologically classified as benign mixed tumors (BMT) based on the prevalence of benign cells under WHO classification. The BMT cells resembled epithelial components in the luminal and/or myoepithelial region associated with mesenchymal cells surrounded by fibrous tissue. Four samples originating from solid nodules were characterized as CC, mainly composed of increasing papillary projections and arrangements of tumor cells in solid sheets, cords, or nets in a histology analysis. Another four samples, considered as large solid nodules, being. Finally, the last four samples collected from the largest solid nodules were classified histologically as squamous cell carcinoma (SCC), a special type of mammary carcinoma according to WHO classification. Histological parameters described the prevalence of islands and cords of epithelial cells with the formation of keratin pearls.

All tumor samples were able to generate primary cells that could be cultured *in vitro* for 10 consecutive passages. During the processing of enzymatically prepared cells, fragments of initial tumor were kept to generate cell cultures. No culture substrates, including matrigel, extracellular matrix, and dried type I collagen, were used. These substrates commonly enhance cell attachment. Specimens attached grew at a good rate initially and were successfully passaged for BMT, CC, SC, and SCC. In this study, fibroblasts were controlled by culture, trypsinization, and attachment during consecutive passages.

Morphological features of p53 and HER2 immunostaining for original tumours (Figure 1A and 1B) and respective cell cultures (Figure 1C and 1D) were scored according to intensity and number of positive cells/mm^2.

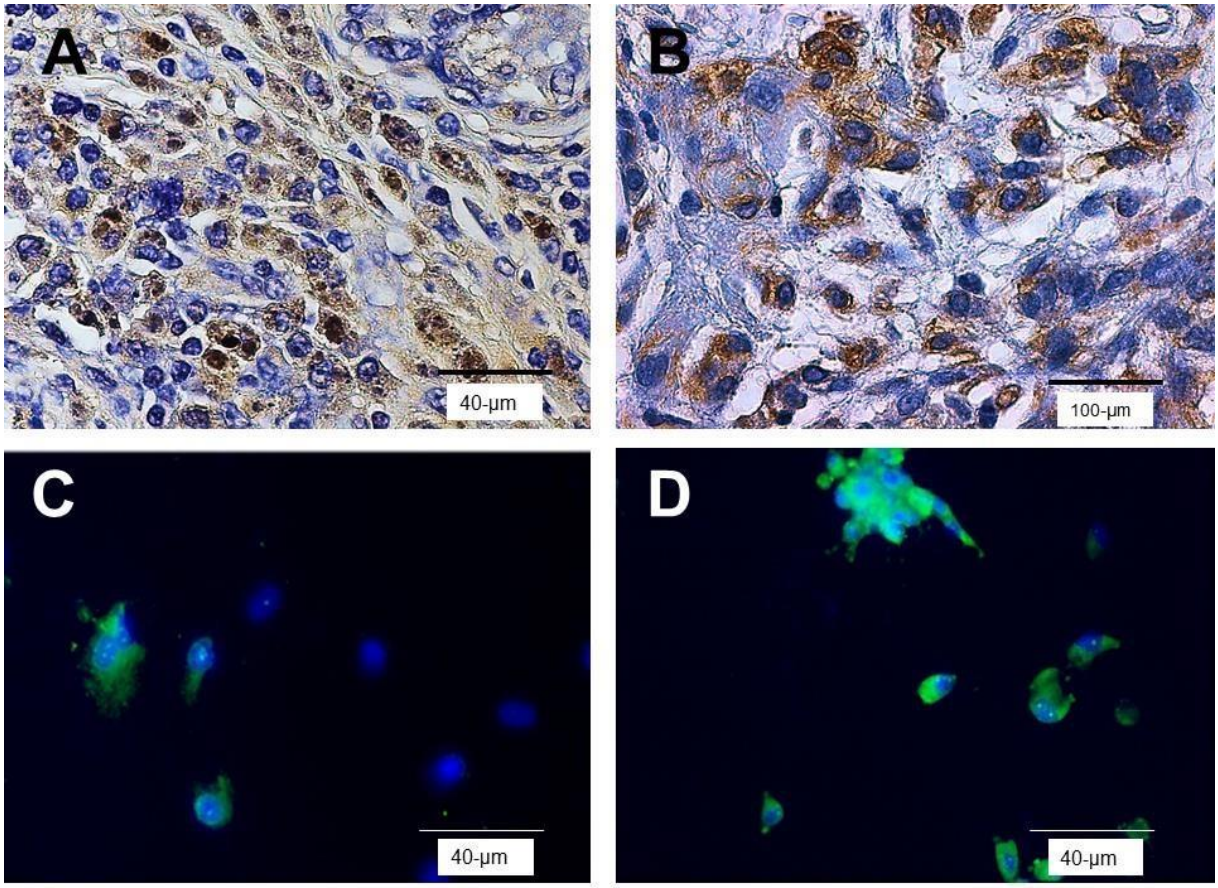

Figure 1 Images features of p53 and HER2 cell markers in CMT original tumors by immunohistochemistry assay (A and B) and derived cell culture by immunocytochemistry assay (C and D). Magnification 40x and 100x, respectively.

The BMT samples showed the lowest number of p53-reactive cells in comparison to CC and SC tumors (Figure 2). However, SCC showed an absence of p53-reactive cells in all samples analyzed (Figure 2). In comparison, BMT-, CC-, SC- and SCC-derived cell cultures showed higher p53 reactivity than the original tumors (Figure 2). SCC had numerous p53-reactive cells in comparison to the original tumor, as demonstrated (Figure 2). HER2-reactive cells were detected at the lowest level in CC samples and did not differ among BMT, SC, and SCC histological sections (Figure 2). BMT-, CC-, SC-, and SCC-derived cell cultures

exhibited an increase in HER2-reactive cells under *in vitro* conditions compared with the original tumors (Figure 2). In relation to the original tumor, HER2-reactive cells were higher in number among SCC-derived cell cultures (Figure 2).

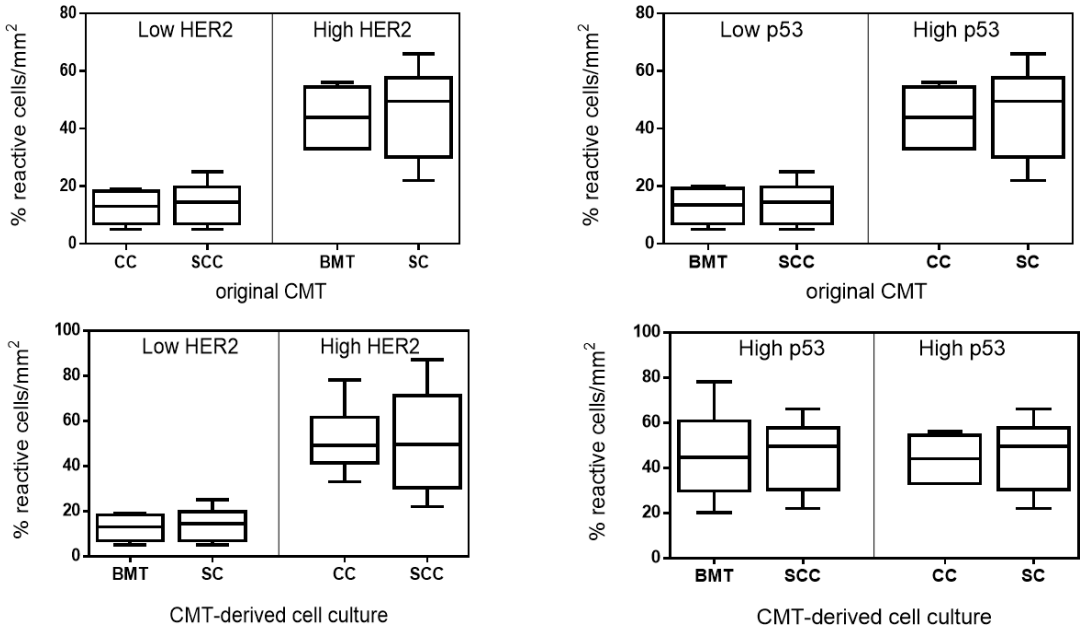

Figure 2 Association of HER2 and p53 expression in canine mammary tumors. * $p \leq 0.005$ considered statistically different.

4. Discussion

Cancer prevalence is increasing among domestic animals, particularly among dogs and cats, similar to humans, due at least in part to greater longevity through more effective and efficient control of infectious and other diseases (Sleeckx et al 2011; Shafieed et al 2013). In addition, it is known that dogs are one of the most susceptible animals to developing spontaneous CMT, leading to the use of dogs as models for comparative studies of human cancers (Shafieed et al 2013). In this study, clinical and pathological variables, such as age, breed, and tumor size, and location, were not considered.

There are only a limited number of reports available in the CMT literature demonstrating the role of HER2 and p53 in an isolated perspective (Lee et al 2004; Linjawai et al 2004; Kumaraguruparan, Prathiba and Nagini 2006; Bertagnolli et al 2009; Hsu et al 2009; Kim et al 2011; Ressel et al 2013). Herein, BMT revealed a prevalence of vimentin-reactive cells similar to previous studies in humans and dogs (Ackand 2003). Overall, WHO classification facilitated the direct comparison of CMT analysis results described elsewhere to those of this investigation (Hellmén 2000; Thompson et al 1999; Kumaraguruparan, Prathiba and Nagini 2006; Andrade et al 2010; Sleeckx 2011; Kim et al 2011; Ressel et al 2013). However, recently, a particular pathologist's view has been documented for CMT classifications, which renders it impossible to correlate results on CMT from different sources (Goldschmidt et al 2011).

The great advantage of using CMT-derived culture cells is an unlimited source of homogenous self-replicating material, free of contaminating stromal cells, and often easily cultured using a simple standard method (Hellmén et al 2000; Lacroix and Leclerq 2004; Krol et al 2012; Krol et al 2009). There are general environmental differences between cancer cells growing *in vitro* and heterogeneous tissue *in vivo* (Hellmén et al 2000). The ability of cell lines to reflect precisely both the phenotype and genotype of parental histology still remain questionable (Wensman et al 2008). In a previous study, primary cell cultures derived from CMT showed up to 94 % similarity to the original tumor in all the genes expressed (Krol et al 2009). In fact, the upregulation of some genes during cell culture can be explained by the action of growth stimulatory factors (Lacroix and Leclerq 2004). In this study, to avoid this phenomenon, growth-promoting supplements, such as hormones, commonly used for this purpose were not employed (Lacroix and Leclerq 2004).

Wild-type p53, a tumor suppressor protein, functions as a transcriptional regulator, genomic stabilizer, an inhibitor of cell cycle progression, and facilitators of apoptosis (Kastan et al 1991). Studies have shown that CMT with wild-type p53 gene expression have a better prognosis than those expressing the mutant protein (Lee et al 2004; Linjawi et al 2004; Bertagnolli et al 2009). Moreover, mutant p53 protein is more stable than wild-type p53 and can be easily analyzed by IHC and immunocytochemical techniques (Ressel et al 2013). According to previous studies, the expression of p53 protein was low in BMT, medium in CC and SC, and the protein was not expressed at all in original SCC tumor samples (Lee et al 2004; Linjawi et al 2004; Kumaraguruparan, Prathiba and Nagini 2006; Bertagnolli et al 2009). However, under *in vitro* culture, all CMT-derived cell lines showed the same 40 % of p53-reactive cells. Most of the p53 labeling was detected in the cytoplasm (data not shown).

In fact, p53 can be divided into two forms in mammalian cell lines, cytoplasmic p53 and nuclear p53 (Lee et al 2004). In the nucleus, p53 abets autophagy mainly by interacting with its target, damage-regulated autophagy modulator (Kastan et al 1991). In contrast, cytoplasmic p53 has been discovered to inhibit autophagy without the assistance of its role as a transcriptional factor (Lee et al 2004). In this study, p53 overexpression in the cytoplasm of MCT-derived cells might be caused by blocking of the autophagy process, as demonstrated before.

HER2 expression is correlated with poor prognosis in human breast cancer, whereas in CMT, the role of this protein is poorly understood and controversial (Citri and Yarden 2006; Ressel et al 2013). HER2 overexpression seems not to identify a subgroup of CMT and/or correlate with poor prognosis as in human breast cancer (Hayden et al 2011). Recent data show significant benefits from combining an anti-HER2 agent with endocrine therapy in HER2-positive cancer in women (Ressel et al 2013). Little is known about how HER2 interacts in female dogs, despite some recent reports describing a positive correlation between malignancy and the overexpression of HER2 in histological samples, in contrast to a previous study (Kim et al 2011). Nevertheless, an increase in survival among dogs was observed when overexpression of the HER2 protein was associated with a single silent nucleotide polymorphism in the canine ERBB2 gene (Hsu et al 2009; Hayden et al 2011; Kim et al 2011). Herein, the same p53 staining pattern was observed for the HER2 protein among CMT-derived cell cultures, which could help to understand the pathways of HER2 activation in dogs.

5. Conclusions

These results open new perspectives on using CMT-derived cell cultures to understand the cross-talk between HER2 and p53, and how this interaction leads to the survival of long-term cultures *in vitro*. In conclusion, because genetic programs can be turned on/off in a controlled way, CMT-derived cell culture may be a useful biological model for epigenetic studies.

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Conflict of Interest

The authors declare no conflict of interests.

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