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c-erbB-2 expression and nuclear pleomorphism in canine mammary tumors

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Abstract

The objective of the present investigation was to study the expression of c-erbB-2 and MIB-1 and try to associate them with morphological features of the cell such as nuclear pleomorphism, mitotic count and histological grade in a series of 70 canine mammary gland tumors, 22 of them benign and 48 malignant. Tumors were collected at the Veterinary Hospital of UFMG (Brazil) and the Veterinary Faculty of Porto University (Portugal). c-erbB-2 expression was determined according to the guidelines provided by the manufacturer of the HercepTest system and nuclear pleomorphism, mitotic count and histological grade according the Elston and Ellis grading system. The HercepTest is the FDA-approved *in vitro* diagnostic test marketed by Dako. It is a semi-quantitative immunohistochemical assay used to determine overexpression of HER2 protein (human epidermal growth factor receptor) in breast cancer tissue. MIB-1 expression was also evaluated in 28 malignant tumors. Seventeen (35.4%) of the malignant tumors were positive for c-erbB-2 expression, which was positively associated with nuclear pleomorphism ($P < 0.0001$), histological grade ($P = 0.0017$) and mitotic count ($P < 0.05$). Nuclear pleomorphism also showed a positive association with MIB-1 index ($P < 0.0001$). These results suggest that some of the biological and morphological characteristics of the tumor are associated in canine mammary gland tumors, as also reported for human breast cancer. It was also possible to show that the immunoexpression of c-erbB-2 can be a factor in mammary carcinogenesis. This fact opens the possibility of using anti-c-erbB-2 antibodies in the treatment of canine mammary tumors.

Key words
- Canine mammary tumors
- Nuclear pleomorphism
- Breast cancer
- c-erbB-2 expression
- MIB-1 expression

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Introduction

A number of proto-oncogenes and oncogenes have been investigated in breast cancer due to their relevant roles in breast carcinogenesis, as well as for their prognostic value. Proto-oncogenes are normal genes that are usually responsible for the regulation of cell proliferation, but in mutated form promote neoplastic transformation. c-erbB-2, c-MYC, c-RAS, and RR1 are examples of some oncogenes involved in mammary carcinogenesis.

The proto-oncogene c-erbB-2 is located on chromosome 17 and encodes a 185-kDa transmembrane glycoprotein with tyrosine kinase activity, which has sequence homology with the epidermal growth factor receptor (1). Since the initial report by Slamon et al. (2), which showed an association between the amplification of this gene and poor prognosis in breast cancer, several other studies have been conducted, and c-erbB-2 overexpression was detected in about 10 to 20% of invasive breast carcinomas. It is not clear, however, whether this overexpression is significantly associated with overall decrease survival and disease-free survival.

The demonstration of a high frequency of c-erbB-2 overexpression in *in situ* ductal carcinomas of the breast suggests that it could be an early event in breast carcinogenesis in humans (3-8); c-erbB-2 overexpression has also been detected in dog mammary tumors (9,10).

The assessment of nuclear pleomorphism involves a cytological evaluation of the structural features of tumor nuclei that can provide information about the grade of malignancy of the tumor. Since nuclear pleomorphism does not reflect the growth pattern of the tumor, it is applicable to all types of mammary carcinoma. However, histological grading does take into consideration the growth pattern, mitotic index and cytological features of differentiation of carcinomas (11,12).

In human breast cancer, histological grade has been correlated with prognosis (11-13) and with c-erbB-2 expression (14-16). c-erbB-2 expression has also been correlated with nuclear alterations in several studies of human breast cancer (14,17-19), showing the existence of a correlation between a morphological and a biological factor.

Since mammary tumors are common in dogs (20), many attempts have been made to improve their histopathological classification in order to predict their biological behavior. In addition to studies using the classic diagnostic tools, some investigators have reported the role of c-erbB-2 expression (9,10).

The aim of the present study was to determine if there is an association of the molecular characteristics of tumor biology (c-erbB-2 expression and MIB-1 index) with morphological aspects (nuclear pleomorphism, mitotic count and histological grade) in canine mammary gland tumors.

Material and Methods

Tumors

Seventy tissue samples of mammary tumors were obtained from bitches that underwent surgery at the Veterinary Hospital or from the Pathology Sector of the Department of Clinical and Surgical Veterinary Medicine, Veterinary School of UFMG (Brazil), and from the Veterinary Faculty of Porto University and Institute of Molecular Pathology and Immunology of Porto University, Porto (Portugal). Seventeen of the cases studied at the Veterinary Hospital of UFMG were followed up for three years between 1996 and 2000. At 3-month intervals after surgery, the owners were asked about the health status of their animals. In case of death, they were asked to indicate the date. This information was compared and supplemented with data from the patient files. Tumors were classified according to the veterinary and human nomenclature (Table 1) (21,22). We used human diagnostic criteria and human classification in order to compare the lesions of the two species.

Immunohistochemical analysis

Serial 4-µm sections were cut from normal canine mammary tissue and from tumor samples previously fixed in buffered 10% formalin and paraffin embedded. Immunohistochemistry was performed using the streptavidin-biotin-peroxidase complex

method (Ultra Vision Large Volume Detection System antipolyvalent, HRP - ready to use - Lab. Vision, Fremont, CA, USA) with a polyclonal antibody against c-erbB-2 (Dako, Glostrup, Denmark) and with the monoclonal antibody MIB-1 (Immunotech, Marseilles, France). Heat-induced antigen retrieval using the Dako Antigen Retrieval Solution (Dako) was previously performed in a wet bath for 20 min. The reagents were applied using an automatic immunohistochemical system (Lab Vision Autostainer, Model LV-1) as described elsewhere (23). As a positive control we used human breast cancer tissue known to express c-erbB-2 and MIB-1. Negative controls were assessed using normal serum as the primary antibody.

The intensity of staining for c-erbB-2 was scored by two observers according to the HercepTest scoring system (Table 2). The HercepTest is the FDA-approved *in vitro* diagnostic test marketed by Dako. It is a semi-quantitative immunohistochemical assay used to determine overexpression of HER2 protein (human epidermal growth factor receptor) in breast cancer tissue. MIB-1 staining was confined to nuclei with a diffuse staining pattern. The intensity of nuclear staining varied among tumors, presumably reflecting not only inherent differences among the tumors but also minor differences in specimen handling. The proliferative index was calculated by counting the positive nuclei in a total of 1000 tumor cells using the Leica-Qwin (Cambridge, UK) image system.

Histological grading and nuclear pleomorphism

Forty-two and forty-five cases were available for histological grade and nuclear pleomorphism assessment in 4-µm tissue sections stained with hematoxylin and eosin. Seventeen tumors (9 ductal carcinomas and 8 metaplastic carcinomas) were followed up over a 3-year period. This period of time is equivalent to 12 years of follow-up in humans (24). Briefly, a 2-year-old dog has the same biological age of a human with 24 years of life and after this time, for each year of canine life 4 years are added for equivalence to human age.

The histological grade was established by one observer according to the grading system proposed by Elston and Ellis (11) (Table 3). This system takes into account tubule formation, nuclear pleomorphism and mitotic index. When more than 75% of the tumor area is composed of well-established tubules a score of 1 point is given. Two points are appropriate for tumors with 10 to 75% of the area showing tubule formation. A score of 3 is assigned to cases with tubular

Table 1. Histological classification of canine mammary tumors according to veterinary and human criteria (21,22).

Veterinary criteria	N	%	Human criteria	N	%
Mixed tumor	12	17.1	Mixed tumor	12	17.1
Simple adenoma	5	7.1	Adenoma	5	7.1
Complex adenoma	3	4.3	Adenomioepithelioma	3	4.3
Duct papilloma	2	2.9	Papilloma	2	2.9
Carcinoma in benign tumors	18	25.7	Metaplastic carcinoma	18	25.7
Simple carcinoma	2		Apocrine carcinoma	2	2.9
Simple carcinoma	5		Ductal carcinoma	17	24.3
Complex carcinoma	2	2.9			
Solid carcinoma	10	14.2			
Tubule papillary carcinoma	5	7.1	Papillary carcinoma	5	7.1
Osteosarcoma	3	4.3	Osteosarcoma	3	4.3
Squamous cell carcinoma	3	4.3	Squamous cell carcinoma	3	4.3
Total	70	100	Total	70	100

Table 2. Classification of c-erbB-2 expression determined by the HercepTest (Dako) immunochemical method.

Score	Assessment of c-erbB-2 protein overexpression	Staining pattern
0	Negative	No staining is observed, or membrane staining in less than 10% of the tumor cells
1+	Negative	A faint barely perceptive membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of the membrane
2+	Positive	A weak to moderate complete membrane staining staining is observed in 10% of the tumor cells
3+	Positive	A strong complete membrane staining is observed in more than 10% of the tumor cells

formation corresponding to 10% of the tumor or less.

For nuclear pleomorphism, the score of 1 is appropriate when the nuclei are small, with little increase in size in comparison with normal breast epithelial cells, and have regular outlines and a uniformity of nuclear chromatin. A score of 2 is assigned when the cells are larger than normal, have open vesicular nuclei with visible nucleoli, and show moderate variability in size and shape. A score of 3 corresponds to marked variation in size and shape, especially when very large and bizarre nuclei are vesicular with prominent, and often multiple nucleoli.

Table 3. Summary of semi-quantitative methods for assessing histological grades in mammary carcinomas as proposed by Elston and Ellis (11).

Feature	Score
Tubule formation	
Most of the tumor (>75%)	1
Moderate degree (10-75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in cell size and variability	2
Marked variation	3
Mitotic counts*	
0-7	1
8-16	2
>17	3
Microscope Olympus BX-40	
Objective	40X
Field diameter (mm)	0.55
Field area (mm^2)	0.239

*Number of mitoses per 10 fields at the tumor periphery.

Mitotic activity is best assessed at the periphery of the tumor, where active growth is most likely, and a minimum of 10 fields are assessed. Strict criteria for identification of mitotic figures were employed, in accordance with van Diest et al. (25). No hyperchromatic or pyknotic nuclei were counted, because these cells may be undergoing necrosis or apoptosis (11). Scoring was originally performed using an Olympus BX-40 microscope fitted with a 10X eyepiece and a 40X objective, which provide a field area of 0.239 mm^2. Up to 7 mitoses per 10 fields are scored as 1 point, 8-16 are scored as 2 points and more than 17 are scored as 3 points.

Histological grade was obtained by summing the scores of these three parameters and classified as follows: 3-5 points: grade I, well differentiated; 6-7 points: grade II, moderately differentiated; 8-9 points: grade III, poorly differentiated.

Statistical analysis

Data concerning c-erbB-2 expression, MIB-1 index, nuclear pleomorphism, mitotic count, and histological grade were analyzed statistically by the Mann-Whitney test. The Spearman rank correlation test was used to compare survival, the influence of c-erbB-2 expression and nuclear pleomorphism adjusted for prognostic factor, from the same sections. Relative risks were presented with 95% confidence intervals.

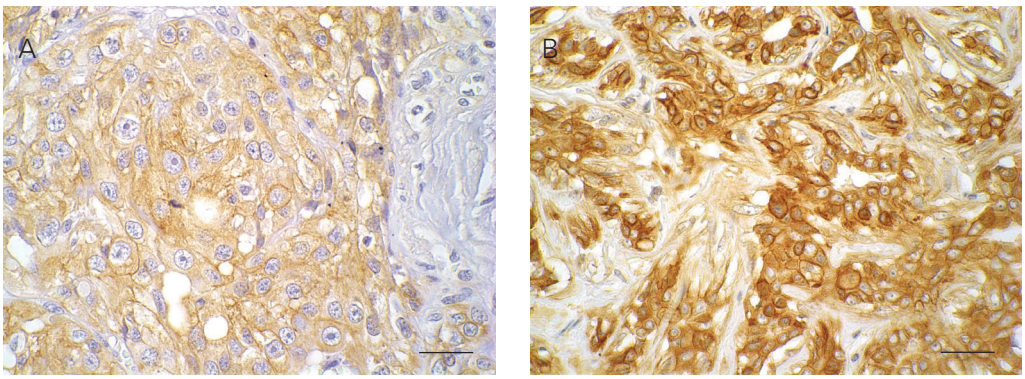

Figure 1. Immunohistochemical reaction to c-erbB-2 protein in canine mammary tumors. *A*, Invasive ductal carcinoma. A weak to moderate complete membrane staining is observed in 10% of tumor cells (2+ score). *B*, Carcinoma with metaplasia. A strong complete membrane staining is observed in more than 10% of the tumor cells (3+ score). Streptavidin-biotin-peroxidase staining. Magnification: A and B, 150X. Bar = 100 µm.

Results

c-erbB-2 expression in canine mammary gland tumors

c-erbB-2 protein was overexpressed in 17 of 48 (35.4%) malignant tumors, with a 2+ score in 64.4% (11/17; Figure 1A) and score 3+ in 25.3% (6/17; Figure 1B). c-erbB-2 protein expression was considered to be negative with a 0 score in 12/48 (25%) and with a 1+ score in 19/48 (39.6%) malignant tumors. None of the 22 benign tumors were positive for c-erbB-2 and 9 (40.9%) were considered to be negative with a 1+ score (Table 4).

c-erbB-2 expression and nuclear pleomorphism

Forty-five malignant tumors were analyzed for nuclear pleomorphism, which was classified as proposed by Elston and Ellis (11). A significant correlation between c-erbB-2 expression and nuclear pleomorphism was observed when the Spearman test was applied ($P < 0.0001$).

Twenty-nine of 45 tumors (64.4%) did not express c-erbB-2 protein, whereas 16 tumors (25.6%) expressed it. Of these 16 positive cases, 9 (56.2%) were classified as 2+ and 43.8% (7 cases) as 3+.

Nuclear pleomorphism classified as grade 3 was detected in 37.5% of the tumors (6/16), 2 and 4 of which had 2+ and 3+ scores for c-erbB-2, respectively. Of 16 tumors (25.6%) with nuclear pleomorphism classified as grade 2, 9 expressed c-erbB-2 protein (56.3%). Of these 9 cases, 7 were classified as 2+ and 2 as 3+. Only one tumor with nuclear grade 1 expressed c-erbB-2 protein and was classified as 3+.

c-erbB-2 expression and histological grade

Forty-two malignant tumors were analyzed. There was a highly significant correlation between c-erbB-2 expression and histological grade ($P = 0.0017$).

Twenty-nine of 42 (69%) cases were negative and 31% (13/42) expressed c-erbB-2 protein, with a 2+ score in 53.8% (7/13) and a 3+ score in 46.2% (6/13).

The majority of tumors were assigned to grade II (42.8%, 18/42), that was positive to c-erbB-2 in 38.5% (5/13), with 4 and 1 scored 2+ and 3+, respectively. The distribution of cases was similar for grade I (12/42 cases, 28.6%) and grade III (12/42 cases, 28.6%). However, the first was positive to c-erbB-2 in 38.5% (5/13), with 1 and 4 scored 2+ and 3+, respectively. The second was positive to c-erbB-2 in 23% (3/13), with 2 and 1 scored 2+ and 3+, respectively.

c-erbB-2 expression and mitotic count

c-erbB-2 protein expression. Forty-eight malignant tumors were analyzed. There was a significant correlation ($P = 0.0213$) between c-erbB-2 expression and mitotic count. Thirty-one of 48 cases (64.6%) did not express c-erbB-2 protein and 17 cases (25.4%) expressed it. Of 17 cases with c-erbB-2 protein overexpression, 11 (64.7%) were scored 2+ and 6 were scored 3+ (25.3%).

Number of mitoses. Most tumors (25/48, 52.1%) received a score of 1 (less than 7 mitoses). Twenty-five from 48 (52.1%) that was positive to c-erbB-2 in 41.2% (7/17), with 2 and 5 scored 2+ and 3+, respectively. Mitotic counting with score 2 was positive to c-erbB-2 in 43.5% (4/17), with 4 scored 2+. Already, mitotic counting with score 3 had been positive to c-erbB-2 in 35.3% (6/17),

Table 4. c-erbB-2 score in benign and malignant tumors determined by immunohistochemistry.

	Negative		Positive		Total
	0	1+	2+	3+	
Malignant tumors	12	19	11	6	48
Benign tumors	13	9	–	–	22

with 5 and 1 scored 2+ and 3+, respectively.

Nuclear pleomorphism and MIB-1 index

Samples of 28 malignant tumors were analyzed. There was an extremely significant relation ($P < 0.0001$) between nuclear pleomorphism and MIB-1 index (Ki-67 expression).

Twenty-two of 28 tumors (78.5%) showed Ki-67 expression in more than 10% of neoplastic cells and in these cases nuclear pleomorphism was classified as 2 (8/22, 26.4%) or 3 (14/22, 63.3%). Six of the 22 cases (21.5%) expressed Ki-67 antigen in less than 10% of tumor cells, with nuclear grade classified as 2 (3 cases) and 3 (3 cases). Detailed results are shown in Table 5.

Prognostic analysis. Seventeen malignant tumors were analyzed, eight metaplastic mammary carcinoma and nine ductal mammary carcinoma. Univariate analysis showed that c-erbB-2 expression ($r = 0.441$, $P = 0.0764$) and nuclear pleomorphism ($r = -0.295$, $P = 0.2499$) were not prognostic factors.

Discussion

Overexpression of c-erbB-2 protein detected by immunohistochemical methods in formalin-fixed and paraffin-embedded specimens has been reported in 10% (25) to 43.7% (8) of human breast cancers, as opposed to 19.1% and 74% (9) of canine cases in series reported by Ahern et al. (9) in a study on 23 dogs and by Rungsipipat et al. (10) in a study on 47 dogs, respectively. In our series we detected c-erbB-2 overexpression in 17 (35.4%) of 48 cases. Perhaps this large range was due to the number of cases and/or to the different methods used to detect c-erbB-2 expression.

Table 5. Frequency of the characteristics of malignant canine mammary tumors.

Characteristic	N	Mean	Standard deviation	Median
c-erbB-2 expression	48	1.20	0.96	1.00
MIB-1 index	28	24.60	18.08	21.00
Histological grade	42	1.95	0.79	2.00
Nuclear pleomorphism	45	2.46	0.54	2.00
Mitotic count	48	1.70	0.84	1.00

Different results concerning c-erbB-2 expression have been reported in several studies on human breast cancer using different methods and number of cases, such as: 15% (17), 16% (14), 24% (26), 27.5% (15), 32.5% (27), 34% (28), 36.3% (18), 37.4% (29), 38% (30), 45% (16), 47.1% (31). The variations in c-erbB-2 positivity in human breast cancer were analyzed taking into account the different methods used for evaluation. Thus, it is necessary to standardize the methods in order to improve the results obtained, which will contribute to future associations and interpretations.

In our series, the correlation between c-erbB-2 expression and histological grade was statistically significant ($P = 0.0017$). This correlation has been observed in several studies on human breast cancer with amplification (14,15,30) and expression (16,31) of c-erbB-2. However, this expression was not associated with histological grade in some studies (8,29,30,32,33). Overexpression of c-erbB-2 protein was detected in 38.5% (5/13) of grade II cases, in 23% (3/13) of grade III cases, and in 38.5% (5/13) of grade I cases. Similar results was found by Tsuda et al. (14).

Of the two components of histological grade, c-erbB-2 expression was correlated with both nuclear pleomorphism and number of mitotic figures. These results suggest that c-erbB-2 expression is associated with cell growth and/or cell division both in canine mammary carcinomas and in human breast carcinoma (14,19,26,28).

Yang et al. (19) demonstrated a positive correlation between nuclear grade and c-erbB-2 overexpression ($r = 0.172$, $P = 0.0117$), and between nuclear grade and MIB-1 index ($r = 0.485$, $P < 0.0001$). In their study, nuclear grade was evaluated accord-

ing to the criteria of the National Surgical Adjuvant Study of Breast Cancer (NSAS-B) protocol. However, in the current study nuclear pleomorphism was evaluated according to Elston and Ellis (11), and we obtained similar results regarding the correlation between nuclear pleomorphism and c-erbB-2 expression ($P < 0.0001$), nuclear pleomorphism and MIB-1 index ($P < 0.0001$), and mitotic count and c-erbB-2 expression ($P = 0.0213$). Tsuda et al. (14) obtained similar results regarding the correlation between mitotic count and c-erbB-2 expression, although they graded mitotic figures according to the system of Bloom-Richardson (12).

Perhaps these results indicate that nuclear pleomorphism and c-erbB-2 expression are associated with the tumor behavior, for example aggressiveness in accordance with cellular proliferation.

Univariate analysis showed that nuclear pleomorphism and c-erbB-2 expression were not predictors of survival. However, in view of the small number of cases studied in the present investigation, the significance of this finding is limited. While these factors were not associated with survival in several studies of human breast cancer (13,14,25,27,33, 34), this association was observed in other studies (15,27,34,35). Genestie et al. (13), in a study involving 825 cases, did not show a positive correlation between nuclear pleomorphism of Elston and Ellis (11) and survival.

In the present series, we showed positive correlations between biology (c-erbB-2 expression and MIB-1 index) and morphology (nuclear pleomorphism, mitotic count and histological grade) in canine mammary gland tumors. These results indicate that the morphology and biology of canine mammary malignant tumors are closely linked, just as in human breast cancer. Therefore, these results reinforce the idea that spontaneous canine mammary tumors can be used as a model to study the mammary carcinogenic process and may be relevant to the study of human breast cancer (36-38).

The expression of immunoreactive c-erbB-2 in canine mammary tumors could be an important factor in canine mammary carcinogenesis. In humans, c-erbB-2 expression has been implicated in the initiation of breast cancer, and has even been used as an oncogene-targeted therapy (39,40). This fact opens up the possibility of using anti-c-erbB-2 antibodies for the treatment of canine mammary tumors. In addition, further investigation is necessary to assess the value of the c-erbB-2 marker as a predictive factor in mammary cancer.

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