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Assessment of cell proliferation and prognostic factors in canine mammary gland tumors

[*Avaliação da proliferação celular e fatores prognósticos em tumores mamários caninos*]

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ABSTRACT

Three methods for the analysis of cell proliferation, mitotic index/10 high-power fields (10 HPF), mitotic index/four sets of 10 HPF (40 HPF), and MIB-1 index were evaluated in a series of canine mammary gland tumors, as well as the possible correlation between them. Fifty-six canine mammary gland tumors, including 23 benign and 33 malignant, were studied. In addition, the prognostic impact of mitotic index/10 HPF, and histological malignancy grade were evaluated in 17 malignant tumors, being seven ductal and 10 metaplastic carcinomas. The three methods used to evaluate cell proliferation were correlated with the prognostic impact of mitotic index/10 HPF and histological malignancy grade. The results showed a strong association between mitotic figure counts and MIB-1 index ($P<0.0001$). A correlation was observed between mitotic count per 40 HPF and MIB-1, and between mitotic index per 10 HPF and 40 HPF ($P<0.05$). Moreover, histological malignancy grade and mitotic figure counts were excellent prognostic factors during three-year follow-up ($P<0.05$). There was a correlation between the three methods used for the evaluation of cell proliferation and prognostic factors as observed in human breast cancer studies.

Keywords: canine mammary tumors, MIB-1 index, mitosis counting

RESUMO

Avaliaram-se três métodos de proliferação celular, índice mitótico/10 campos de grande aumento (10 CGA), quatro vezes 10 CGA (40 CGA) e índice de marcação por MIB-1, em uma série de tumores mamários caninos, e as possíveis correlações entre estes métodos. Foram estudados 56 tumores mamários caninos, 23 benignos e 33 malignos. Foi também avaliado o impacto prognóstico do índice mitótico (10 CGA) e o grau histológico maligno em 17 tumores malignos, sete carcinomas ductais e 10 carcinomas metaplásicos. A correlação entre os três métodos para avaliar a proliferação celular e o impacto prognóstico do índice mitótico por 10 CGA e o grau histológico maligno foi realizada. Os resultados mostraram que existe uma forte associação entre contagem de mitose e o índice de marcação por MIB-1($P<0,0001$) e correlação entre contagem de mitoses em 40 CGA e índice de marcação por MIB-1 e entre índice mitótico em 10 CGA e 40 CGA ($P<0,05$). Observou-se correlação entre os três métodos de avaliação da proliferação celular e os fatores prognósticos semelhante aos estudos de câncer de mama humano.

Palavras-chave: tumor mamário canino, MIB-1, contagem de mitoses

INTRODUCTION

Different methods for the evaluation of cell proliferation in neoplasms have been developed over recent years, including morphometry, flow cytometry, nucleotide radiolabeling, and immunohistochemistry. However, these methods are limited by technical and/or economical aspects; therefore, mitotic figure counting continues to be the most traditional and the simplest method to estimate cell proliferation in human breast cancer and is one of the

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components of the Bloom-Richardson grading system (Bloom and Richardson, 1957; Elston and Ellis, 1991). Additional reproducible methods have been applied to more accurately investigate cell proliferation (Quinn and Wright, 1990; Linden et al., 1992). Among these methods, Ki-67 has been the most widely studied marker for the evaluation of cell proliferation in tumors. This antibody reacts with nuclear antigens present during the G1, S, and G2 phases and during mitosis, but is absent during G0. The MIB-1 antibody recognizes the Ki-67 antigen in formalin-fixed paraffin-embedded tissue after antigen retrieval and has been applied to the study of human breast cancer (Weidner et al., 1994; Schmitt and Ferreira, 1995; Lehr et al., 1999).

Determination of the MIB-1 proliferation index in human breast cancers has an important prognostic impact (Schmitt and Ponsa, 2000). Breast cancers presenting a high proliferation index are associated with a higher rate of recurrence, low survival and shorter postoperative disease-free period. The use of a proliferation index as a predictive factor of chemotherapy has shown good initial results (Marinho et al., 1997). A positive correlation has also been observed between MIB-1 and tumor size, histological grade, p53 expression, and aneuploidy (Schmitt and Ferreira, 1995).

In human patients, estrogen and progesterone receptor determination is an established procedure in the routine management of patients with breast cancer and is used as a predictive factor for adjuvant hormone therapy (Andersen and Poulsen, 1989; Pertschuck et al., 1996).

Mammary neoplasms account for 25% and probably for as much as 50% of all neoplasms in bitches (Moulton et al., 1970). Some canine mammary gland tumors are hormone dependent as demonstrated by the fact that early ovariohysterectomy will reduce their incidence to 0.05% (Moulton et al., 1970). The relevance of canine mammary gland tumors as a therapeutic model for human breast cancer is still undefined. These tumors do not seem to respond to chemotherapy at the same extent as human breast cancer. As the biology and molecular pathology of canine mammary gland tumors is further clarified, these tumors may become a better model for human breast cancer (Vail and MacEwen, 2000).

Since mammary tumors are common in dogs, many attempts have been made to improve their histopathological classification in order to more precisely predict their biological behavior. In addition to studies involving the classic histopathological diagnosis, some publications have shown a certain relevance of cell proliferation parameters (Lohr et al., 1997; Pena et al., 1998; Sarli et al., 1999). The aim of the present study was to compare three different methods for the evaluation of cell proliferation, mitotic index per 10 high-power fields (10 HPF) (Elston and Ellis, 1991; 1998), mitotic index per four sets of 10 HPF (40 HPF) (Baak, 1990), and MIB-1 index, in 56 canine mammary gland tumors. The prognostic impact of mitotic figure counting per 10 HPF and histological malignancy grade and type was also evaluated in 17 out of 33 malignant tumors.

MATERIAL AND METHODS

Fifty-six canine mammary tumors (Table 1) were obtained from bitches that underwent surgery for mammary tumors at the Departamento de Clinicas e Cirurgia da Escola de Veterinária da UFMG, Brazil; at Faculdade de Veterinária da Universidade do Porto, Portugal; and at IPATIMUP, Porto, Portugal. Seventeen malignant tumors were obtained from bitches that underwent surgery for mammary tumors at the Escola de Veterinária da UFMG and were followed up for three years between 1996 and 2000. Every three months after surgery, the owners were asked regarding the health status of their animals, including the occurrence and time of death. This information was compared and supplemented with data from the files of dogs. Tumors were classified according to veterinary and human nomenclature (Rosen and Oberman, 1993; Misdorp et al., 1999) (Table 1). Human diagnostic criteria and human classification were used to compare the lesions of the two species.

Table 1. Histological types of canine mammary tumors according to veterinary and human nomenclature*

Veterinary classification	N	%	Human classification	N	%
Mixed tumor	12	21.4	Mixed tumor	12	21.4
Complex adenoma	3	5.4	Adenomyoepithelioma	3	5.4
Simple adenoma	6	10.7	Adenoma	6	10.7
Duct papilloma	2	3.6	Papilloma	2	3.6
			Secretory carcinoma	2	3.6
Simple carcinoma	7	12.5	Apocrine carcinoma	1	1.8
			Ductal carcinoma	9	16.0
Complex carcinoma	1	1.8			
Solid carcinoma	4	7.2			
Carcinoma in benign tumor	10	16.0	Metaplastic carcinoma	10	18.0
Tubulopapillary carcinoma	3	5.4	Papillary carcinoma	3	5.4
Squamous cell carcinoma	3	5.4	Squamous cell carcinoma	3	5.4
Anaplastic carcinoma	2	3.6	Micropapillary carcinoma	2	3.6
Osteosarcoma	2	3.6	Osteosarcoma	2	3.6
Fibrosarcoma	1	1.8	Fibrosarcoma	1	1.8
Total	56	100	Total	56	100

*According to Rosen and Oberman (1993) and Misdorp et al. (1999).

Antibody assays were performed on serial 4-µm sections of normal and neoplastic canine mammary tissue fixed in 10% buffered formalin and embedded in paraffin. The streptavidin-biotin-peroxidase method was used[1]. Antigen retrieval was performed in a double boiler[2]. The reagents were applied using an automated immunohistochemistry system[3] as described elsewhere (Cassali et al., 2001; 2007). Sections from cases of human mammary neoplasms known to express MIB-1 were used as positive control. For the negative control, the primary antibody was replaced with normal serum. MIB-1 staining was confined to nuclei presenting a diffuse staining pattern. MIB-1 proliferative activity was assessed with an image analysis system[4], determining the percentage of positive cells among 1,000 tumor cells (MIB-1 index).

Seventeen out of 33 malignant tumors were available for histological examination, including seven primary ductal mammary carcinomas and 10 metaplastic mammary carcinomas. These cases were followed up over a period of three years. According to Lebeau (1953), this period corresponds to a 12-year follow-up period in humans. Mammary tissue sections were cut at 4µm from each tumor and stained with hematoxylin and eosin. Each tumor was graded by one author, except for the mitotic figure counting per 10 HPF, which was performed by Observers 1 and 2, according to the criteria proposed for human breast tumors (Elston and Ellis, 1991; 1998) (Table 2). These criteria included tubule formation, nuclear pleomorphism, and mitotic count.

Table 2. Summary of the semiquantitative method for assessing histological grade in mammary carcinoma*

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

*According to Elston and Ellis (1991; 1998)
**Assessed as number of mitoses per 10 fields at the tumor periphery.

[1]Ultra Vision Large Volume Detection System antipolyvalent, HRP – Ready to Use, Lab Vision
[2]Retrieval Solution, Dako
[3]Lab Vision Autostainer Model LV-1
[4]Leika-Qwin

For mitotic count, points were originally assigned using an Olympus BX-40 microscope equipped with a 10X eyepiece and a 40X objective, providing a field area of 0.239mm^2. Under and including seven mitoses per 10 fields scored 1 point, 8-16 scored 2 points, and more than 17 scored 3 points (Elston and Ellis, 1991; 1998). The overall tumor grade was calculated by summing the scores obtained for each category, resulting in a possible total score of 3-9 (Elston and Ellis, 1991; 1998). The tumor grade was then classified as follows: 3-5 points: grade I – well differentiated; 6-7 points: grade II – moderately; differentiated, and 8-9 points: grade III – poorly differentiated. This method of evaluating tumor differentiation is essentially based on a subjective assessment of morphological features. Therefore, it is advisable to validate the results, if possible.

Two statistical methods were used: Pearson's correlation coefficient was calculated between all proliferation markers and log rank correlation was used to compare survival and the influence of histological grade, mitotic count per 10 HPF, and histological type (Campos-Filho and Franco, 1990) adjusted for prognostic factor. Relative risks are reported as 95% confidence intervals (CI). Survival curves were derived from Kaplan-Meier estimates. Interobserver agreement for mitotic count was tested using pairwise and generalized kappa statistics (Fleiss, 1981). The divisions of kappa statistic providing "benchmarks" for strength of agreement were determined as previously described (Landis and Koch, 1977).

RESULTS

The mean age of the dogs at the time of surgery was 8.6 years (range: three to 15-year-old). The material consisted of 41% (23/56) benign and 59% (33/56) malignant tumors. Table 3 shows means, standard deviations, and ranges of cell proliferation rates determined by the observers. A positive correlation was observed between mitotic figure count per 10 HPF, mitotic count per 40 HPF, and MIB-1 index.

Table 3. Proliferative indices of canine tumors obtained by the observers

Marker	Mean		Standard deviation		Range		Units
	Observer1	Observer2	Observer1	Observer2	Observer1	Observer2	
10 HPF	1.3	1.2	1.5	2.1	0-94	0-116	Mitosis/10 HPF
40 HPF	0.9	1.1	2.9	1.9	0-100	0-120	Mitosis/40 HPF
MIB-1	18.5		17.7		1-76		%1000 cells

Observer 1: student; Observer 2: senior pathologist.

According to Observer 1, a significant association was observed between mitotic count per 10 HPF and mitotic count per 40 HPF (P=0.0308, r=0.2042) and between mitotic count per 40 HPF and MIB-1 index (P=0.0235, r=0.3023). The results obtained by Observer 2 were considered extremely significant (P<0.0001, r=0.5436). However, both observers found an extremely significant correlation (P<0.0001) between mitotic figure count per 10 HPF and MIB-1 index.

Pairwise kappa values of interobserver agreement for mitotic count per 10 HPF and 40 HPF were substantial, with values of 0.77 and 0.62, respectively.

A positive correlation was observed between mitotic count, histological grade, and survival. A significant correlation was observed between mitotic count and survival by the two observers (Observer 1: r= –0.6047, P=0.0101; Observer 2: r= –0.5403, P=0.0251). Irrespectively of the method used by the observer for scoring mitotic activity, the results showed an unbalanced distribution, probably due to the inexperience of Observer 1 (Table 4). Observer 1 scored 52.8% (n=9) of the cases as 1, 23.6% (n=4) as 2, and 23.6% (n=4) as 3. According to Observer 2, the percentage of tumors scored as 1 decreased to 41.7% (n=7), whereas 35.3% (n=6) and 23.6% (n=4) were scored as 2 and 3, respectively.

Table 4. Distribution of mitotic score and histological grade of the cases of canine tumors according to the observers

	Observer 1 N (%)	Observer 2 N (%)
Mitotic score		
1	9 (52.8)	7 (41.1)
2	4 (23.6)	6 (35.3)
3	4 (23.6)	4 (23.6)
Histological grade		
I	8 (47)	6 (35.3)
II	5 (29.4)	7 (41.1)
III	4 (23.6)	4 (23.6)

Observer 1: student; Observer 2: senior pathologist.

An extremely significant association was observed between histological grade and survival (Observer 1: r= –0.7946, P=0.0001; Observer 2: r= –0.7520, P=0.0005). The results showed an unbalanced distribution of the cases between the observers (Table 4). Observer 1 graded 47% (n=8), 29.4% (n=5), and 23.6% (n=4) of the cases as I, II, and III, respectively. According to Observer 2, the percentage of tumors graded as I decreased to 35.3% (n=6), whereas the percentage of those graded as II increased to 41.1% (n=7) and the remaining 23.6% (n=4) were graded as III.

Univariate analysis showed that histological grade (P=0.0029) and mitotic count (P=0.032) were good prognostic factors irrespectively of the observer, in spite of the fact that the number of cases, 17, would have taken too long. However, four animals (scored as 3 upon mitotic counting) died within one year, in accordance with both observers (Fig. 1a). Four cases graded as III had died within nine months (corresponding to three years in humans), in accordance with observers 1 and 2 (Fig. 1b). There was a highly significant correlation between histological grade and prognosis: survival was less likely in cases with poor differentiation compared to well-differentiated tumors (Fig. 1b).

The mean age of the dogs that were followed up at the time of surgery with metaplastic mammary carcinoma was 9.4-year-old (range: 5 to 13-year-old), corresponding 53.5-year-old (range: 36 to 68-year-old) in women. The mean age of dogs with ductal carcinoma was 7.9-year-old (range: 3 to 12-year-old), corresponding to 43.1-year-old (range: 28 to 64-year-old) in women.

Univariate analysis showed that histological type (P=0.022) was a good prognostic factor, despite the fact that the number of cases, 17 (10 metaplastic carcinomas, seven ductal carcinomas), would have taken too long.

The mean follow-up periods were 26.7 and 10.4-month-old (range: 3 to 36-month-old).

Table 5 shows that 1/3 (33.3%) and 1/4 (25%) metaplastic mammary carcinoma cases graded as II by observers 1 and 2, respectively, survived three years (corresponding to 12 years in humans). However, 6/7 (85.7%) and 5/6 (83.3%) metaplastic mammary carcinoma cases graded as I by observers 1 and 2, respectively, survived three years (12 years). It was observed that no cases of ductal mammary carcinoma graded III survived, and that no case graded II by Observer 1 and 1/3 (33.3%) of cases graded II by Observer 2 survived for three years (12 years).

Considering 15 months of survival (corresponding to five years according in humans), 8/10 (80%) and 1/7 (14.3%) cases of metaplastic and ductal carcinoma, respectively, survived during this period. The 3-year survival (12-year survival) was 6/10 (60%) for metaplastic carcinoma and 2/7 (28.6%) for ductal carcinoma (Fig. 1c).

Table 5. Distribution of cases with 5 and 12-year survival regarding histological type and grade*

Histological type	N	Histological grade	5-year survival		12-year survival			
			Observer 1	Observer 2	Observer 1	Observer 2	Observer 1	Observer 2
Metaplastic	10	I	7	6	7	6	6	5
		II	3	4	1	2	1	1
Ductal	7	I	1	0	1	0	1	0
		II	2	3	0	1	0	1
		III	4	4	0	0	0	0

*According to Lebeau 1953) according to observer.
Observer 1: student; Observer 2: senior pathologist.

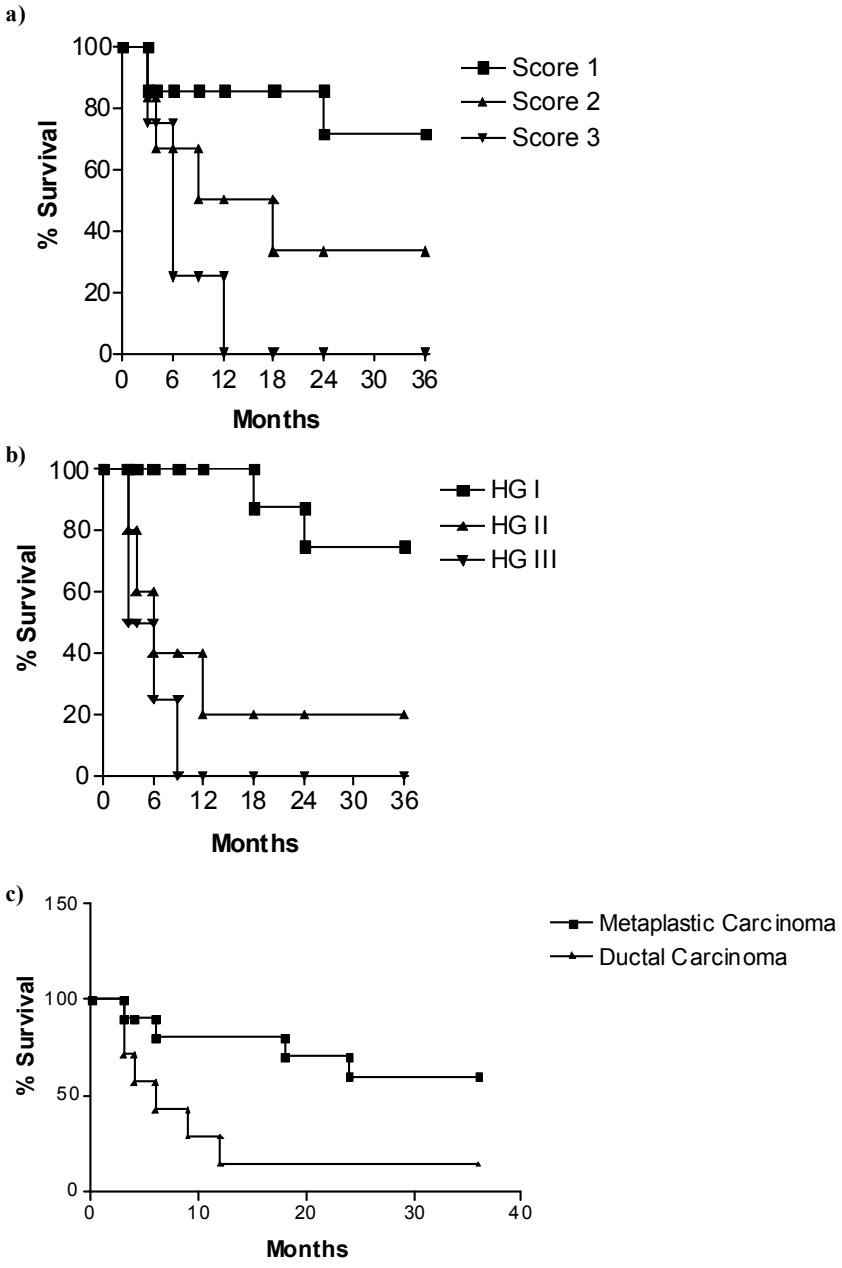

Figure 1. a) Association of mitotic score with survival in 17 malignant tumors; b) association of histological grade (HG) with survival in 17 malignant tumors; c) association of histological type with survival in metaplastic and ductal carcinomas.

DISCUSSION

Cell proliferation rates have been increasingly used as prognostic factors in patients with breast cancer (Le Doussal et al., 1989; Van Diest et al., 1992; Weidner et al., 1994; Schmitt and Ferreira, 1995). Most of the methods used to estimate tumor proliferation rates, even those used in studies on human cancer, are time consuming, costly, and their routine application is difficult (Weidner et al., 1994; Schmitt and Ferreira, 1995; Isola et al., 1990; Rudolph et al., 1998). Over recent years, the estimation of growth fractions by immunocytochemical staining with antibodies against proliferation and associated antigens has been widely applied to human

(Weidner et al., 1994; Schmitt and Ferreira, 1995; Aranda and Laforga, 1997; Rudolph et al., 1998; Lehr et al., 1999) and canine tumors (Lohr et al., 1997; Peña et al., 1998; Sarli et al., 1999; Cassali et al., 2002).

Counting mitotic figures by light microscopy is still a relatively rapid and low cost method for the estimation of tumor cell proliferation (Weidner et al., 1994; Aranda and Laforga, 1997). In addition, some investigators have found mitotic figure content to be a good prognostic indicator in human patients with breast carcinoma (Le Doussal et al., 1989; Clayton and Hopkins, 1993). In the present study, there was a significant correlation between mitotic figure count per 10 HPF and prognosis, with survival being less likely in cases with a score of 3 compared to those with a score of 1 (Fig. 1a). Mitosis counting has been criticized because pathologists disagree on what constitutes a mitotic figure. Van Diest et al. (1992) have shown that good interobserver agreement can be achieved when strict criteria are applied to the recognition and counting of mitotic figures. In the present study, this strict criterion was used and accurate results were obtained in all correlations among proliferation markers, despite the inexperience of one observer. These findings indicate that a certain degree of experience in diagnostic pathology, in addition to strict criteria, is mandatory for reliable mitotic figure counting.

A positive correlation was observed between the MIB-1 index and mitotic count. This correlation has been reported in several studies using Ki-67 to assess proliferation in human breast cancer (Weidner et al., 1994; Schmitt and Ferreira, 1995; Aranda and Laforga, 1997; Rudolph et al., 1998; Lehr et al., 1999). The extremely strong correlation observed between MIB-1 index and mitotic count per 10 HPF supports the view that MIB-1 is an effective indicator of proliferative activity in sections of canine mammary gland tumors, as confirmed in previous reports of human breast cancer (Schmitt and Ferreira, 1995; Aranda and Laforga, 1997). However, mitotic figure count per 10 HPF is a simple, relatively rapid and low-cost method for estimating tumor cell proliferation that could be used as a laboratory routine procedure or in veterinary studies on canine mammary tumors, confirming the results of Aranda and Laforga (1997) for human breast cancer.

Tumor size and the presence or absence of axillary lymph node metastases have been recognized as important prognostic factors in human breast cancer (Nemoto et al., 1980; Lelle et al., 1987; Sarli et al., 1999), but other factors such as histological grade (Nemoto et al., 1980; Lelle et al., 1987; Sarli et al., 1999) and type (Henson, 1988) have been reported to be equally, if not, more important.

The present results confirm that histological grade is correlated with the outcome of canine mammary tumors. Only 2/8 (25%) or 1/6 (16.6%) of cases scored as grade I died within two years (eight years, Fig. 1b) (Lebeau, 1953), in accordance with observers 1 and 2, respectively. In contrast, all cases scored as grade III died during the first year (four years, Fig. 1b) (Lebeau, 1953). These results suggest that survival is lower in poorly differentiated cases compared to well-differentiated tumors, as observed in human studies (Bloom and Richardson, 1957; Nemoto et al., 1980; Elston and Ellis, 1991; Henson et al., 1991). In their original study on 1830 patients, Elston and Ellis (1991) showed a strong correlation between histological grade and prognosis.

Metaplastic carcinoma accounts for less than 5% of all human breast cancers, whereas in dogs metaplastic carcinoma is observed from 18% to 30% of cases (Allen, 1940; Cassali, 2000) (Table 1). Additionally, lesions occurring in dogs may have a more benign clinical course (Fidler and Brodey, 1967). With equivalent ages ranging from 36 to 68 years (Lebeau, 1953), with an average age of 51.2 years, this age was very close to that found in human metaplastic breast carcinoma studies (Huvos et al., 1973; Kaufman et al., 1984; Chhieng et al., 1998). The overall 5-year survival rate was 8/10 (80%) (Table 5). Rates of 40% to 65% have been reported in the literature for human cases (Huvos et al., 1973; Kaufman et al., 1984; Wargotz and Norris, 1989). The overall 12-year survival (Lebeau, 1953) rate was 6/10 (60%), suggesting that canine mammary tumors behave in a more benign clinical way than human lesions (Huvos et al., 1973). However, the number of cases was small and therefore the significance of such finding is limited.

Ductal carcinoma represents the largest group of human malignant mammary carcinomas,

accounting for 65% to 80% of cases (Schmitt and Ponsa, 2000) of human mammary carcinomas, and is the most frequent malignant tumor in dogs (Cassali, 2000) (Table 1). In ages ranging from 28 to 64 years, with an average age of 41.2 years, in human subjects, ductal carcinoma is common between from 55 to 60-year-old (Rosen and Oberman, 1993). In ductal carcinoma cases, the overall 5- and 12-year survival rate was 1/7 (14.3%) (Table 5). In a study with humans, Elston and Ellis (1991) found overall 5- and 12-year survival rates of 53.7% (983/1830) and 6.6% (122/1830), respectively. The 12-year survival rate was 6/10 (60%) in cases of metaplastic carcinoma and 2/7 (28.5%) in cases of ductal carcinoma (Fig. 1c). These results suggest that ductal carcinoma has a worse prognosis than metaplastic carcinoma in dogs, similarly to human breast cancer (Chhieng et al., 1998).

This study emphasizes the importance of mitotic count and histological grade and type for assessing the prognosis of canine mammary tumors and indicates that the criteria used for this assessment should be well standardized and controlled. In contrast to the MIB-1 index, mitotic count per 10 HPF is less expensive, requires no additional technology, applies to most histological types, and can be carried out on formalin-fixed tissue sections.

The present study showed that mitotic count per 10 HPF and histological grading as proposed by Elston and Ellis (1991; 1998) can be used as prognostic factors of canine mammary gland tumors, as well as of human breast cancer. Thus, mitotic count, histological grade and type, immunohistochemical staining with MIB-1, and survival of the dogs in this study seem to be comparable with these features in human breast cancer. The present results suggest that canine mammary tumors might be used as a model for the study of the overall mechanisms of mammary carcinogenesis (Cassali et al., 1999; 2001).

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